REMARKS

No amendments or cancellation have been made to claims 75-129. Accordingly, these claims are currently under Examiner's reconsideration.

For the Examiner's convenience, Applicants' remarks are presented in the same order in which they were raised in the Office Action.

Information Disclosure Statement

The Examiner states that Reference 64 (Rote Liste 1999) previously submitted in the September 4, 2007 Supplemental Information Disclosure Statement was not considered because it is not in English and no translation has been provided. Applicants respectfully submit that an English translation of the reference is provided in the Supplemental Information Disclosure Statement submitted herewith. Consideration and initial of this reference, as well as other references cited in the Supplemental Information Disclosure Statement is respectfully requested.

Claim Interpretation

The Examiner acknowledges that claims 90, 93, 94, 111, and 113, which recite a method of administering paclitaxel at amount that is "about 1% to about 20%," "about 1% to about 10%," or "about 1% to about 5%," comply with 35 U.S.C. § 112, second paragraph. The Examiner, however, states that the claims encompass administration of paclitaxel at any dose, because the specification "does not define to what extent the recited 'about' modifies the claimed ranges." Applicants respectfully disagree with this interpretation.

"Generally claim terms should be construed consistently with their ordinary and customary meanings, as determined by those of ordinary skill in the art." *Merck & Co., Inc., v. Teva Pharmaceuticals USA*, Inc., 395 F.3d 1364, 1370 (Fed. Cir. 2005). The ordinary meaning of the term "about" is "approximately." *Id.* at 1369. Given the ordinary meaning of the term "about," Applicants respectfully submit that claims 90, 93, 94, 111, and 113 should not be interpreted to encompass administration of paclitaxel at any dose.

Applicants further submit that the Examiner improperly relies on pages 8 to 9 of the specification for support of the interpretation of the term "about" to encompass administration of paclitaxel at any dose. Specifically, the term "from about 1% to less than about 98% of the amount of pharmacologically active agent conventionally administered" in the specification does not define the term "about" as encompassing administration of paclitaxel at any dose. Instead, it merely provides a range of the administration amount whose lower limit is "about 1%" and upper limit is "about 98%." Similarly, the present claims, for example claim 90, recite a range of the administration amount whose lower limit is "about 1%" and upper limit is "about 20%."

Claim Rejections – 35 USC § 101

Claims 75-89 are rejected under 35 U.S.C. § 101, as allegedly lacking substantial utility. The Examiner states that the methods of administering sub-therapeutic doses of paclitaxel fail to provide an immediate benefit to the public, because the biological effects alleged in the specification using the claimed methods are not apparent, because necessary experiments would be required to identify or reasonably confirm a "real world" context of sub-therapeutic dose in the treatment of cancer, and because clear and convincing proof is required to show that the claimed methods have therapeutic use in treating human patients. Applicants respectfully traverse this rejection.

In accordance with § 2107.01 of the Manual of Patent Examining Procedure (M.P.E.P.), "[o]ffice personnel must be careful not to interpret the phrase 'immediate benefit to the public' . . . to mean that products or services based on the claimed invention must be 'currently available' to the public in order to satisfy the utility requirement. *See, e.g., Brenner v. Manson*, 383 U.S. 519, 534-35, 148 USPQ 689, 695 (1966). Rather, any reasonable use that an applicant has identified for the invention that can be viewed as providing a public benefit should be accepted sufficient, at least with regard to defining a 'substantial' utility." § 2107.01 of M.P.E.P.

In the present application, Applicants have identified the reasonable use of the claimed invention for the benefit to the public. Specifically, the specification discloses that, if plasma levels of paclitaxel are maintained at 0.01-0.05 μg/ml over a period of a week or longer, significant benefit

is obtained in the treatment of cancers responsive to paclitaxel. Page 18, lines 18-21 of the Specification. Although the plasma levels recited in the claims, namely, 0.01-0.05 μg/ml, are below the levels that are high enough to be cytotoxic to the tumor cells as disclosed in the specification, they are above the levels that are required for an anti-angiogenic effect on human endothelial cells. Belotti et al., *Clin. Cancer Res.* 2:1843-9 (1996)(*Exhibit* 1)(paclitaxel inhibits endothelial cell migration and invasiveness with an IC_{50} of 0.4 nM and 0.5 nM respectively, equivalent to 0.33 ng/ml and 0.41 ng/ml respectively). *See also* Bocci et al., *Cancer Research*, 62:6938-43 (2002)(*Exhibit 2*)(vascular endothelial cell growth inhibition and apoptosis was induced using continuous 144 hour long-term exposure of paclitaxel with an IC_{50} value range of 25-143 pM, equivalent to 0.021 ng/ml to 0.12 ng/ml); Wang *et al.*, *Anti-Cancer Drugs*, 14:13-19 (2003) (*Exhibit 3*)(paclitaxel at ultra-low concentrations of 0.1 pM to 100 pM, equivalent to 0.083 pg/ml to 0.083 ng/ml, inhibit angiogenesis).

The Examiner states that "in the absence of clear and convincing proof that the claimed methods have therapeutic use in treating human patients," the claimed invention lacks a substantive utility. Applicants respectfully disagree. According to the M.P.E.P., to establish utility, Applicants do not have to provide evidence that the "asserted utility is true 'beyond a reasonable doubt'" or to a "statistical certainty." *See* M.P.E.P. § 2107.02 VII. Evidence is sufficient if it leads one skilled in the art to conclude that "the asserted utility is more likely than not true." *Id.* (emphasis in original). Applicants respectfully submit that, in the present case, the evidence is sufficient to lead one skilled in the art to conclude that it is more like than not that the claimed method provides significant benefit in the treatment of cancers responsive to paclitaxel.

Applicants further submit that the term "sub-therapeutic dose levels" used in the present application refers to levels of pharmacologically active agent (such as paclitaxel) that are lower than *conventionally accepted levels* considered essential for successful treatment of the infirmity when the pharmacologically active agent is administered by *conventional means*. Page 7, lines 9-15 and 21-26 of the specification. It does not mean that the amount being administered is not therapeutically effective as the Examiner states.

Accordingly, Applicants respectfully request that the rejection under 35 U.S.C. § 101 be withdrawn.

Claim Rejections - 35 USC § 112 (1st Paragraph)

Claims 75-89

Claims 75-89 are rejected under 35 U.S.C. § 112, first paragraph, as allegedly failing to comply with the enablement requirement.

The Examiner states, "[b]ecause the claims lack a substantial utility as set forth above, they also lack enablement *a priori*." As discussed above, claims 75-89 have satisfied the utility requirement under 35 U.S.C. § 101. Accordingly, Applicants respectfully submit that the claims are enabled.

Furthermore, Applicants note that claims 75-89 are directed to a method of administering paclitaxel, wherein the plasma level of paclitaxel in the subject is maintained at 0.01-0.05 μg/ml over a period of 7 days or more. One skilled in the art is enabled by the specification to administer paclitaxel to a subject and maintain the plasma level of paclitaxel at 0.01-0.05 μg/mL over a period of 7 days or more.

The Examiner's attention is further directed to Merchan et al., *International Journal of Cancer*, 113:490-498 (2005) (*Exhibit* 4). Merchan et al. shows that a prolonged intravenous infusion of paclitaxel at 10 mg/m^2/day for 6 consecutive days, which provided a steady-state plasma level of around 10 nM (0.0083 μg/ml), had anti-endothelial effects. Such effects were potently enhanced when in combination with a Cox-2 inhibitor.

Accordingly, Applicants respectfully request that the rejection of claims 75-89 under 35 U.S.C. § 112, first paragraph be withdrawn.

Claims 90-129

Claims 90-129 are rejected under 35 U.S.C. § 112, first paragraph, for allegedly failing to comply with the enablement requirement. The Examiner alleges that the specification, while being enabled for administering paclitaxel at or near "conventional doses" over 7 days or more, does not reasonably provide enablement for administering paclitaxel at dose levels 1-20% or 1-10% of the conventional dose of paclitaxel. Applicants respectfully traverse.

The Examiner points to Herben *et al.*, which discuss irinotecan and conclude that "the optimal administration schedule of irinotecan in the clinic is still uncertain." The Examiner also points to Blumenreich *et al.*, which discuss etoposide and conclude that "low-dose continuous oral etoposide is a well-tolerated but ineffective regimen in non-small cell lung cancer." The Examiner further points to Sørensen *et al.*, which discuss 5-FU and conclude that "long-term continuous infusion of 5-FU had only modest activity in terms of response rate." Applicants respectfully submit that irinotecan, etoposide, and 5-FU are different from paclitaxel in their structure, function, and physical/chemical properties. Accordingly, the references the Examiner relies on are inapplicable to the present claims.

The Examiner also relies on Munoz et al., *The Breast*, 14:466-479 (2005). The Examiner states,

> The authors state that a "major handicap" of "metronomic chemotherapy" is the determination of an optimal biologic "low" dose for any given chemotherapy regimen (page 475). Because many new drugs do not have dose-limiting toxicities or express optimal therapeutic activity below a MTD, this "greatly increases the empiricism associated with using these drugs in clinical trials, and hence the probability of obtaining negative results." (page 475).

Page 12 of the Office Action. Applicants respectfully submit that the paragraphs the Examiner points to relate to new drugs that do not have dose-limiting toxicities or express optimal therapeutic activity below a maximum tolerated dose (MTD). In fact, Munoz et al. acknowledges that "ultra low doses of paclitaxel" have "strikingly potent and selective effects" on human endothelial cells, and that "very low concentrations of most of these drugs [including paclitaxel],

e.g., 25-150 pmol can significantly block proliferation of the endothelial cells, *but only if the cells are exposed continuously*." Page 473. (emphasis in original).

Applicants further submit that the specification provides sufficient teaching that enables one skilled in the art to practice the claimed invention, for example, administering about 1% to about 20% of the conventional dose of paclitaxel and maintaining a therapeutically effective plasma level of paclitaxel over the period of 7 days or more as claimed in claim 90 and regularly administering about 1% to about 20% of the conventional dose of paclitaxel over a period of 7 days or more as claimed in claim 111. The conventional doses of paclitaxel are provided in the specification. *See, e.g.*, page 7, line 28 to page 8, line 1 of the specification. The specification also provide sufficient disclosure that enables one skilled in the art to practice the claimed invention of administering paclitaxel at about 1% to about 20% (or about 1% to about 10%) to a subject over a period of 7 days or more. *See, e.g.* page 5, line 14 to page 9, line 30.

Furthermore, as discussed above, the term "sub-therapeutic dose levels" used in the present application refers to levels of pharmacologically active agent (such as paclitaxel) that are lower than conventionally accepted levels considered essential for successful treatment of the infirmity when the pharmacologically active agent is administered by conventional means. It does not mean that the amount being administered is not therapeutically effective as the Examiner indicates.

In view of the above, Applicants respectfully submit that the specification provides sufficient enablement to the claimed invention and request that the rejection under 35 U.S.C. § 112, first paragraph, be withdrawn.

Claim Rejections - 35 USC § 102(b)

Chang et al.

Claims 90-91, 93-95, 97-98, 100, 103, 105-114, 116-117, 119, 122, and 124-129 are rejected under 35 U.S.C. § 102(b) as allegedly being anticipated by Chang *et al.* ("Chang"). Applicants respectfully traverse this rejection.

To the extent that the Examiner relies on the broad interpretation of the term "about" when making the rejection, Applicants submit that the interpretation is incorrect and thus request that the rejection be withdrawn.

Applicants further submit that Chang does not anticipate any one of the rejected claims. Specifically, Chang discloses a dose escalation study of paclitaxel wherein patients were treated with a one-hour infusion of paclitaxel for 3 weeks starting at 50 $mg/m^2/wk$, with increment of 10 $mg/m^2/wk$ every 5 patients. These dose levels are well above about 1% to about 20% (or about 1% to about 10%) of the conventional doses of paclitaxel, as recited in the present claims.

Furthermore, with regard to claim 90 and its dependent claims, Chang is completely silent about maintaining a therapeutically effective plasma level of paclitaxel throughout the period of 7 days or more. With regard to claim 111 and its dependent claims, Chang is completely silent about regularly administering paclitaxel over a period of 7 days or more.

Applicants therefore respectfully request that the rejection based on Chang be withdrawn.

Klaassen et al.

Claims 90-95, 97-98, 100, 103, 105-108, 110-114, 116-117, 119, 122, 124-127, and 129 are rejected under 35 U.S.C. § 102(b) as allegedly being anticipated by Klaassen *et al.* ("Klaassen"). Applicants respectfully traverse the rejection.

To the extent that the Examiner relies on the broad interpretation of the term "about" when making the rejection, Applicants submit that the interpretation is incorrect and thus request that the rejection be withdrawn.

Applicants further submit that Klaassen does not anticipate any one of the rejected claims. Specifically, Klaassen discloses administration of paclitaxel via a 1-hour infusion on days 1, 8, 15, 22, 29, and 36 (every 50 days) at dose levels of 70 $mg/m^2/wk$, 80 $mg/m^2/wk$, 90 $mg/m^2/wk$, and 100 $mg/m^2/wk$. These dose levels are well above about 1% to about 20% (or about 10%) of the conventional doses of paclitaxel, as recited in the present claims.

Furthermore, with regard to claim 90 and its dependent claims, Klaassen is completely silent about maintaining a therapeutically effective plasma level of paclitaxel throughout the period of 7 days or more. With regard to claim 111 and its dependent claims, Klaassen is completely silent about regularly administering paclitaxel over a period of 7 days or more.

Accordingly, Applicants respectfully request that the rejection based on Klaassen et al. be withdrawn.

Fennelly et al.

Claims 90-95, 97-98, 100, 103, 105-108, 110-114, 116-117, 119, 122, 124-127, and 129 are rejected under 35 USC § 102(b) as allegedly being anticipated by Fennelly *et al.* ("Fennelly"). Applicants respectfully traverse the rejection.

To the extent that the Examiner relies on the broad interpretation of the term "about" when making the rejection, Applicants submit that the interpretation is incorrect and thus request that the rejection be withdrawn.

Applicants further submit that Fennelly does not anticipate any one of the rejected claims. Specifically, Fennelly teaches escalating-doses of paclitaxel at 40 mg/m^2/wk, 50 mg/m^2/wk, 60 mg/m^2/wk, 80 mg/m^2/wk, and 100 mg/m^2/wk administered as a 1-hour infusion in patients with recurrent ovarian cancer. These dose levels are well above about 1% to about 20% (or about 10%) of the conventional doses of paclitaxel, as recited in the present claims.

Furthermore, with regard to claim 90 and its dependent claims, Fennelly is completely silent about maintaining a therapeutically effective plasma level of paclitaxel throughout the period of 7 days or more. With regard to claim 111 and its dependent claims, Fennelly is completely silent about regularly administering paclitaxel over a period of 7 days or more.

Applicants therefore respectfully request that the rejection based on Fennelly et al. be withdrawn.

Claim Rejection 35 USC § 103

Fennelly et al. in view of WO 98/14174

Claims 99, 101, 118, and 120 are rejected under 35 U.S.C. § 103(a) as allegedly being unpatentable over Fennelly as applied to claims 90-95, 97-98, 100, 103, 105-108, 110-114, 116-117, 119, 122, 124-127, and 129 above, and further in view of WO 98/14174 ("WO '174"). Applicants respectfully traverse the rejection.

As discussed above, Fennelly does not teach or suggest administering paclitaxel at about 1% to about 20% (or about 1% to about 10%) of the conventional dose over a period of 7 days or more as recited in the present claims.

WO'174 does not cure the deficiencies of Fennelly. Specifically, WO'174 is cited as allegedly disclosing compositions and methods useful for the *in vivo* delivery of substantially insoluble pharmacologically active agents in the form of suspended particles coated with a protein or in the form of a redispersible dry powder comprising nanoparticles of water-insoluble drug coated with a protein. WO'174 does not teach or suggest administering paclitaxel at about 1% to about 20% (or about 1% to about 10%) of the conventional dose over a period of 7 days or more as recited in the present claims.

Accordingly, Applicants respectfully submit that neither Fennelly nor WO'174 application, alone or in combination, discloses the claimed methods and renders the dosing regimen as currently claimed obvious to one skilled in the art.

Fennelly et al. in view of US 6,458,373

Claims 99-104 and 118-123 are rejected under 35 USC § 103(a) as allegedly being unpatentable over Fennelly as applied to claims 90-95, 97-98, 100, 103, 105-108, 110-114, 116-117, 119, 122, 124-127, and 129 above, and further in view of U.S. Patent No. 6,211,171 (U.S. '171 patent). Applicants respectfully traverse the rejection.

As discussed above, Fennelly does not teach or suggest administering paclitaxel at about 1% to about 20% (or about 1% to about 10%) of the conventional dose over a period of 7 days or more as recited in the present claims.

U.S. '171 does not cure the deficiencies of Fennelly. Specifically, US '171 is cited as allegedly disclosing compositions formulated for local injections comprising a physiological compatible saline solution and may optionally be encapsulated in a slow release delivery vehicle, including a colloidal dispersion system (*e.g.* nanocapsules) or in polymer stabilized crystals. *See* col. 11, lines 1-17. US'171 does not teach or suggest administering paclitaxel at about 1% to about 20% (or about 1% to about 10%) of the conventional dose over a period of 7 days or more as recited in the present claims.

Accordingly, Applicants respectfully submit that neither Fennelly nor U.S. '171 application, alone or in combination, discloses the claimed methods and renders the dosing regimen as currently claimed obvious to one skilled in the art.

Double Patenting Rejection

Claims 90-98, 105-117, and 124-129 are provisionally rejected on the ground of non-statutory obviousness-type double patenting as allegedly being unpatentable over claims 1-14 of co-pending Application No. 11/644,850. The Examiner is respectfully requested to hold this provisional rejection in abeyance until the Office has made a determination of otherwise allowable claims in the present application or in co-pending application No. 11/644,850.

CONCLUSION

In view of the above, each of the presently pending claims in this application is believed to be in immediate condition for allowance. Accordingly, the Examiner is respectfully requested to withdraw the outstanding rejection of the claims and to pass this application to issue. If it is determined that a telephone conference would expedite the prosecution of this application, the Examiner is invited to telephone the undersigned at the number given below.

In the event the U.S. Patent and Trademark Office determines that an extension and/or other relief is required, applicant petitions for any required relief including extensions of time and authorizes the Commissioner to charge the cost of such petitions and/or other fees due in connection with the filing of this document to **Deposit Account No. 03-1952** referencing docket no. 638772000200. However, the Commissioner is not authorized to charge the cost of the issue fee to the Deposit Account.

Dated: May 16, 2008

Respectfully submitted,

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EXHIBIT 1

The Microtubule-affecting Drug Paclitaxel Has Antiangiogenic Activity[1]

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ABSTRACT

Endothelial cell migration is a critical event during angiogenesis, and inhibitors of cell motility can affect the angiogenic process. Paclitaxel (Taxol®), a microtubule-stabilizing antineoplastic cytotoxic drug, inhibits motility and invasiveness of several cell types. The aim of this study was to investigate the effect of paclitaxel on endothelial cell functions and on angiogenesis. *In vivo*, paclitaxel (20–28 mg/kg i.v.) significantly inhibited the angiogenic response induced by tumor cell supernatant embedded in a pellet of reconstituted basement membrane (Matrigel) injected s.c. into C57BL/6N mice. *In vitro*, paclitaxel inhibited endothelial cell proliferation, motility, invasiveness, and cord formation on Matrigel in a dose-dependent manner. The antiangiogenic activity of paclitaxel was not linked to its cytotoxicity, since inhibition of endothelial cell chemotaxis and invasiveness occurred at drug concentrations which did not affect endothelial cell proliferation. Another cytotoxic drug, cisplatin, that inhibited endothelial cell proliferation *in vitro*, did not affect angiogenesis *in vivo*. These data indicate that paclitaxel has a strong antlangiogenic activity, a property that might contribute to its antineoplastic activity *in vivo*.

INTRODUCTION

Agents affecting the cytoskeleton are potential inhibitors of angiogenesis (1). By interfering with cell functions which require an intact cytoskeleton, such as proliferation, migration, and invasion, they can interrupt the angiogenic process at several levels. Microtubule-disrupting agents, such as colchicine and vinblastine, have been reported to inhibit cord formation and endothelial cell migration in an *in vitro* wound-healing assay (2, 3).

The antineoplastic drug paclitaxel affects the cytoskeleton with a unique mechanism of action: at subnanomolar concentrations, paclitaxel favors the assembly of microtubules, reducing the critical concentration of tubulin dimers and the need for cofactors such as guanosine 5′-triphosphate and microtubule-associated proteins. Paclitaxel also stabilizes formed microtubules by shifting the equilibrium between dimers and polymers in favor of the latter and by stabilizing the microtubules against dissociating conditions such as cold and $CaCl_2$. This results in the formation of abnormal cytoskeletal structures: cells exposed to paclitaxel *in vitro* present new microtubules in parallel bundles, asters of mitotic spindles during mitosis, and microtubules not associated with the microtubule organizing centers (4, 5).

The cytotoxic activity of paclitaxel for tumor cells is well known, and preclinical and clinical studies showed it was effective against several tumors, including ovarian, breast, lung, and head and neck carcinomas (5). Other activities of paclitaxel related to different cell functions have been studied as well. The drug inhibits the motility of murine fibroblasts (6), ESb murine T-lymphoma (7), PC-3 prostate carcinoma (8), and A2058 melanoma cells (9). It also inhibits the production of matrix metalloproteinases by tumor cells, thus affecting their invasive behavior (7, 8).

Little is known about the effect of paclitaxel on endothelial cell functions and on angiogenesis. This study investigated the effect of paclitaxel on tumor-induced angiogenesis in *in vitro* and *in vivo* experimental models.

MATERIALS AND METHODS

Drugs and Reagents. Paclitaxel (Taxol®; provided by the Division of Cancer Treatment, National Cancer Institute, Bethesda, MD) was dissolved in 50% polyoxyethylated castor oil (Cremophor EL) and 50% ethanol and diluted in 5% glucose to the indicated concentrations for *in vivo* treatments. For the *in vitro* experiments, a 1000× stock solution of paclitaxel in absolute ethanol was prepared and then diluted in test medium. Cisplatin (Bristol-Myers Squibb, Wallingford, CT) was dissolved in water.

As a source of tumor-derived angiogenic factor, we used the supernatant of murine endothelioma eEnd.1 cells which release the angiogenic factor EDMF[3] in the culture medium (10). The conditioned medium, prepared as described (10), was separated on heparin-Sepharose, and the fractions containing EDMF, corrected for molarity, were used *in vitro* and *in vivo*.

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[1] This study was supported by the Italian Association for Cancer Research, the Italian Research Council (Consiglio Nazionale delle Ricerche, Applicazioni Cliniche della Ricerca Oncologica Project), and the Istituto Superiore di Sanità (AIDS Project). T. D. is a recipient of the Paolo Baffi Fellowship.

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[3] The abbreviations used are: EDMF, endothelioma-derived motility factor; bFGF, basic fibroblast growth factor; MMP-2, matrix metalloproteinase 2.

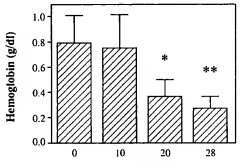

Fig. 1 Effect of paclitaxel on angiogenesis *in vivo*. Pellets of Matrigel containing a tumor-derived angiogenic factor (EDMF) were implanted s.c. in C57BL/6N mice and treated i.v. on days 0 and 2 with the indicated dose of paclitaxel or vehicle. After 4 days, the angiogenic response was evaluated. Data are expressed as hemoglobin content of the pellet (g/dl) and means of the data from at least two independent experiments ($n = 10$–18). Hemoglobin content of pellets containing Matrigel alone was 0.29 ± 0.07 g/dl ($n = 16$). *, $P \leq 0.05$; **, $P \leq 0.005$ (Mann-Whitney *U* test). *Bars*, SE.

Matrigel was kindly provided by Dr. A. Albini (National Cancer Institute, Genova, Italy).

***In Vivo* Angiogenesis Assay.**[4] To study angiogenesis *in vivo*, the method described by Passaniti *et al.* (11) and previously utilized to evaluate inhibitors of angiogenesis (12) was used. Briefly, tumor cell supernatant, prepared as described above, was embedded in a pellet of Matrigel (10 mg/ml, 0.5 ml) along with heparin (16 units/ml) and injected s.c. into male C57BL/6N mice (Charles River, Calco, Italy). Mice received paclitaxel (28 mg/kg, unless otherwise indicated) or cisplatin (4 mg/kg) i.v. immediately before and 2 days after the injection of the pellet of Matrigel. Control mice received the same volume of vehicle.

After 4 days, the angiogenic response was evaluated at autopsy by two blinded observers. Macroscopic evaluation of hemorrhage within and around the Matrigel pellet was graded from − to 4+, depending on the extent and intensity of hemorrhage. The pellet was then removed, and the hemoglobin content was measured with the Drabkin procedure as described (12).

For histological analysis, the Matrigel pellets in combination with surrounding skin and soft tissue were fixed in 10% neutral buffered formalin for 24 h and embedded in paraffin. Five-μm sections were stained with H&E, and the following histological features were independently evaluated and graded by two blinded observers: (*a*) cellularity associated with the surface of the pellet (scored as: 1, scanty; 2, moderate; and 3, abundant); (*b*) cells invading the pellet (scored as above); (*c*) presence of cords and tubules within the pellet (scored as: 0, none; 1, occasional; 2, moderate; and 3, prominent); and (*d*) presence of blood-filled channels and/or lacunae (scored as above). For each sample, the resulting sum was recorded as the final score.

***In Vitro* Assays.** Endothelial cells were obtained from human umbilical vein and cultivated as described (12). A colorimetric assay was used to assay proliferation (12). Briefly, 4×10^3 cells were plated in each well of a 96-well plate. After 24 h, paclitaxel or cisplatin was added at the concentration indicated in "Results"; the drugs were either removed after 4 h or left for the duration of the assay (72 h). After 3 days, the plate was stained with 0.5% crystal violet in 20% methanol, rinsed with water, and air dried. The stain was eluted with a 1:1 solution of ethanol:0.1 M sodium citrate, and absorbance at 540 nm was measured with a Multiscan MC (Titertek; Flow Laboratories, Milan, Italy; Ref. 12). Data are expressed as the percentage of control proliferation (vehicle-treated cells); the IC_{50} (drug concentration causing 50% inhibition) was calculated from the plotted data.

Chemotaxis was assessed using the Boyden chambers and 8-μm pore size, gelatin-coated polycarbonate Nucleopore filters, as described previously (12). For chemoinvasion, the filter was coated with an even layer of Matrigel (0.5 mg/ml), and the assay was conducted as described elsewhere (12). Endothelial cells were detached by a brief exposure to 0.25% trypsin-0.02% EDTA, washed in DMEM with 0.1% BSA, resuspended in this medium at the concentration of $1–2 \times 10^6$ cells/ml, and added to the upper compartment of the Boyden chamber. Paclitaxel or cisplatin was added to the endothelial cells at the indicated concentration and incubated throughout the assay (4 h for chemotaxis, 6 h for chemoinvasion). Data are expressed as the percentage of control migration (vehicle-treated cells); the IC_{50} (drug concentration causing 50% inhibition) was calculated from the plotted data.

To analyze the production of matrix metalloproteinases, subconfluent cultures of endothelial cells were washed three times with serum-free medium and incubated for 20 h in the same medium containing vehicle or paclitaxel. The conditioned medium was then collected and processed for gelatin zymography; the remaining cells were counted. Gelatin zymography was performed essentially as described (13), with 1 mg/ml gelatin in a 10% polyacrylamide-SDS gel. The sample volume was adjusted according to the number of cells. The experiment was repeated twice.

Cord formation on Matrigel was assessed by plating endothelial cells (6×10^4/0.5 ml) on a layer or polymerized Matrigel (270 μl) essentially as described (14). Paclitaxel (1 nM) or vehicle (absolute ethanol) was added to the medium. Photographs were taken with an inverted microscope 48 h after plating.

RESULTS

Paclitaxel Affects Angiogenesis *in Vivo*. s.c. injection of a tumor cell supernatant embedded in a pellet of Matrigel induced a strong angiogenic response in 4 days, with macroscopic hemorrhages around the pellet and a 2.8–3.3-fold in-

[4] Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national (D.L. 116, G.U., Suppl. 40, February 18, 1992) and international laws and policies (EEC Council Directive 86/609, OJ L 358, 1, December 12, 1987; NIH Guide for the Care and Use of Laboratory Animals, NIH Publ. No. 85-23, 1985).

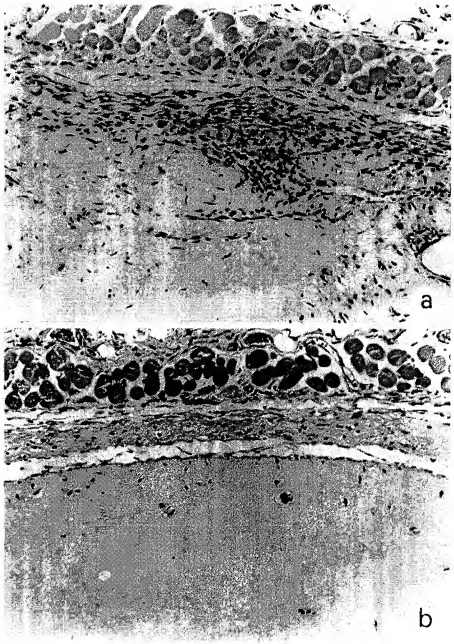

Fig. 2 Inhibition of angiogenesis *in vivo* by paclitaxel: histological analysis of the Matrigel pellets. Infiltration of cells, either single or organized in vascular structures, is evident in the pellets of mice treated with vehicle (*a*), but is greatly reduced in mice treated with paclitaxel (28 mg/kg, *b*). H&E, ×100.

crease in the hemoglobin content compared to pellets containing Matrigel alone. Systemic treatment of mice with paclitaxel resulted in dose-dependent inhibition of the angiogenic response compared to vehicle-treated animals. Inhibition of hemoglobin content was significant at 20 mg/kg ($P < 0.05$) and was maximal at 28 mg/kg, with no effect at 10 mg/kg (Fig. 1). In the same assay, batimastat and tissue inhibitor of metalloproteinase 2, used as reference drugs, caused up to 38 and 75% inhibition of the hemoglobin content of the pellets, respectively (12).

Macroscopic evaluation of the area revealed markedly less hemorrhage surrounding the pellet in the animals treated with 20 and 28 mg/kg paclitaxel: 55% of the vehicle-treated mice presented hemorrhage compared to 22% and 20% of the mice treated with 28 and 20 mg/kg paclitaxel, respectively. Moreover, the mean intensity of hemorrhage scored 1.03 ± 0.3, 0.17 ± 0.08, and 0.25 ± 0.2 for vehicle, 28 and 20 mg/kg paclitaxel, respectively.

Histological analysis of pellets containing the angiogenic factors showed a variable degree of cellularity surrounding and invading the pellets of Matrigel. Cells were scattered singly

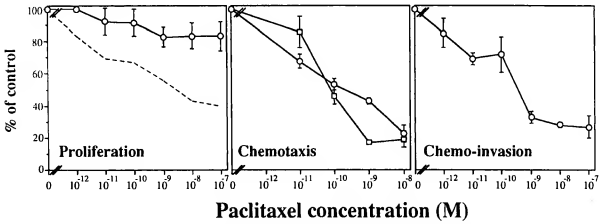

Fig. 3 Effect of paclitaxel on *in vitro* endothelial cell proliferation, motility, and chemoinvasion. The antiproliferative effect of paclitaxel was assessed by treating endothelial cell with the drug for 4 h (○) or 72 h (- - -). Chemotaxis and chemoinvasion were tested in a Boyden chamber using tumor cell-conditioned medium (○) or bFGF (□) as attractants. In these assays, paclitaxel was added to the cells at the concentration indicated and left throughout the assay (4 and 6 h, respectively). Data are expressed as the percentage of proliferation, migration, and invasion compared to control response (ethanol alone), and the means of triplicates from one experiment are representative of at least three. *Bars*, SE.

throughout the Matrigel or organized in thin cords and tubules without a clearly defined lumen, in blood vessels with a recognizable lining of flattened endothelial cells, and in larger blood lacunae, without any apparent cell lining (Fig. 2*a*). A significant reduction in the angiogenic response was observed in the pellets of mice treated with paclitaxel compared to vehicle. The cellularity surrounding and infiltrating the pellets was reduced, and cords, tubules, vessels, and lacunae were less frequent (Fig. 2*b*). The histological score of the cases under study, evaluated as described in "Materials and Methods," ranged from 3 to 11. A strong response (histological score ≥6) was observed in 7 of 10 vehicle-treated mice, but only in 1 of 8 paclitaxel-treated mice ($P = 0.024$, Fisher's exact test).

Paclitaxel Affects Endothelial Cell Functions *in Vitro*. *In vitro* experiments were then performed to study the possible mechanisms of the antiangiogenic activity of paclitaxel. The effect was investigated on endothelial cell functions related to the angiogenic process: proliferation, migration, invasiveness, and cord formation.

The effect of paclitaxel on endothelial cell proliferation was assessed with a colorimetric assay, and endothelial cells were exposed to the drug for either 4 h or 3 days. The short time exposure (4 h) was chosen to compare the effect of paclitaxel on proliferation and motility in the same experimental conditions (see below). When endothelial cells were exposed to paclitaxel for 4 h, only a marginal inhibitory effect was observed (up to 17.3% inhibition; Fig. 3). A strong inhibition of endothelial cell proliferation (up to 60.5% inhibition) was instead observed when cells were exposed to paclitaxel for 3 days (Fig. 3, *dashed line*).

Paclitaxel also inhibited endothelial cell motility response to tumor-derived angiogenic factors. Inhibition was dose dependent, occurring at concentrations as low as 10 pM and reaching 86.2% at 100 nM (Fig. 3). The IC_{50} was 0.4 nM. In one experiment, the angiogenic factor bFGF was used as a chemoattractant, and in this case paclitaxel also inhibited the endothelial cell chemotactic response (Fig. 3).

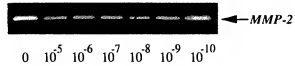

Fig. 4 Zymographic analysis of MMP-2. Endothelial cells were exposed to vehicle or different concentrations of paclitaxel, and volumes of the supernatant corresponding to the same number of cells were electrophoresed on gelatin-containing SDS-polyacrylamide gels. After processing, MMP-2 appears as a clear band of gelatinolytic activity at 72 kDa.

An important event during angiogenesis is the degradation of the basement membrane by endothelial cells to allow their migration into the underlying interstitial matrix. Matrix-degrading enzymes, whose production is stimulated by several angiogenic factors, play an important role in matrix digestion by invading endothelial cells (15). As a model to study endothelial cell invasiveness, we used the chemoinvasion assay in which endothelial cells are induced to digest a layer of reconstituted basement membrane (Matrigel) and to migrate through the filter. Paclitaxel inhibited endothelial cell chemoinvasion, with maximum inhibition (72%) at 10 nM and an IC_{50} of 0.5 nM (Fig. 3). We also found that production of the matrix metalloproteinase MMP-2, evaluated by zymography, was reduced in paclitaxel-treated endothelial cells (up to 37% inhibition at 10 μM, Fig. 4).

The final event during angiogenesis is the organization of endothelial cells in a three-dimensional network of tubes. *In vitro*, endothelial cells plated on Matrigel align themselves, forming cords, already evident a few hours after plating. The

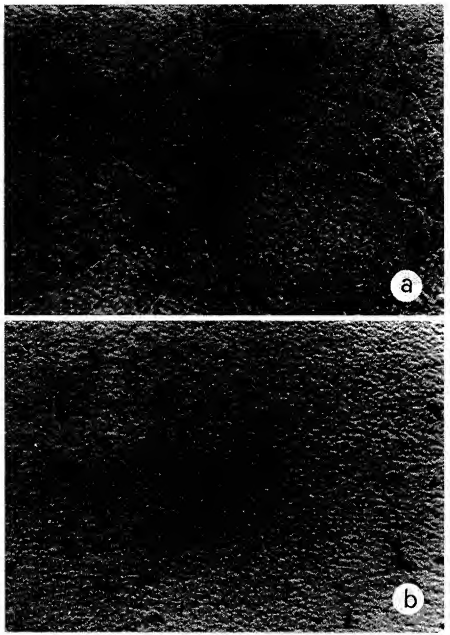

Fig. 5 Cord formation by endothelial cells in Matrigel. Endothelial cells (6×10^4) were seeded on a layer of Matrigel in the presence of vehicle (*a*) or paclitaxel (1 nM, *b*). After 48 h, formed cords were photographed under an inverted microscope (×63). The experiment was repeated three times.

addition of paclitaxel (1 nM) to the assay resulted in complete inhibition of endothelial cell alignment and cord formation (Fig. 5).

Lack of Antiangiogenic Activity of Cisplatin in Matrigel. To rule out a direct association between antiangiogenic and antiproliferative activity, we have studied the effect on endothelial cell functions *in vitro* and on angiogenesis *in vivo* of another cytotoxic drug, cisplatin, known to have a strong antiproliferative effect on different cell types. As expected, cisplatin was very effective in inhibiting endothelial cell proliferation *in vitro*, already after a 4-h exposure IC_{50} was 200 μM (Fig. 6). Inhibition of endothelial cell motility was observed only at toxic concentrations of cisplatin (IC_{50}, 290 μM). Notwithstanding its antiproliferative activity, cisplatin (4 mg/kg) did not inhibit the angiogenic response in Matrigel *in vivo* (Fig. 6).

DISCUSSION

Tumor-induced angiogenesis, the formation of neovessels from preexisting ones, is critical for supporting tumor growth

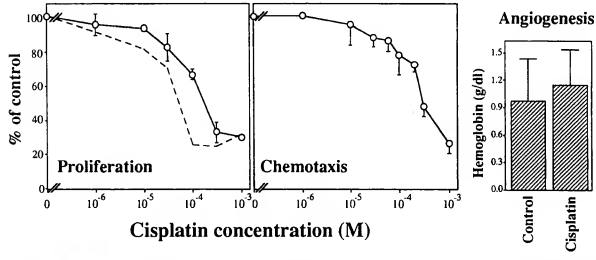

Fig. 6 Effect of cisplatin on endothelial cell functions *in vitro* and angiogenesis *in vivo*. The effect of cisplatin on cell proliferation was assessed by treating endothelial cells with the drug for 4 h (○) or 72 h (- - -). Chemotaxis was tested as described in the legend to Fig. 3. Data are expressed as the percentage of proliferation and migration compared to control response, and the means for data from two independent experiments are represented. Angiogenesis was assayed as described in the legend to Fig. 1. Mice received cisplatin (4 mg/kg) or vehicle i.v. on days 0 and 2. Data are expressed as hemoglobin content of the pellet (g/dl), and the means for data from two independent experiments (n = 12–17) are represented. Hemoglobin content of pellets containing Matrigel alone was 0.31 ± 0.18 g/dl (n = 17). *Bars*, SE.

and progression not only by providing the necessary blood supply but also by allowing metastatic cells into the circulation (16). Promising preclinical studies have shown the antineoplastic activity of angiostatic agents, and several antiangiogenic drugs are currently under investigation in clinical trials. The present study reports that paclitaxel inhibits angiogenesis induced *in vivo* by tumor cell supernatants. This indicates that the antiangiogenic activity of the drug might contribute to its antineoplastic activity, thus tumor growth might be affected not only by direct cytotoxicity for tumor cells but also by inhibition of neovessel formation. This might be particularly important in the case of vascular tumors and tumors characterized by a strong angiogenic activity. Indeed, paclitaxel has been recently described to be active in a Phase II clinical trial in patients with HIV-associated Kaposi's sarcoma, a vascular tumor characterized by a strong angiogenic activity (14, 17).

Paclitaxel inhibited angiogenesis at doses (20–28 mg/kg) that have shown antitumor activity in preclinical studies in mice. Indeed, these doses inhibit the growth of different murine tumors in syngeneic mice and human tumors transplanted in nude mice (18, 19).

Paclitaxel inhibited the proliferation of endothelial cells. The drug did not affect endothelial cell attachment to tissue culture plastic (data not shown), thus ruling out that the inhibition of cell proliferation is indirectly mediated by an effect of paclitaxel on cell adhesion. Given the effect of paclitaxel on endothelial cell proliferation, it was therefore important to study the relationship between antiproliferative and antiangiogenic activity. To this purpose, two approaches were followed.

Studying the effect of the drug on *in vitro* endothelial cell functions relevant to the angiogenic process, we have found that paclitaxel inhibited endothelial cell motility in response to tumor-derived chemotactic factors and to bFGF. Interestingly, endothelial cell motility was inhibited to a greater extent and at lower drug concentrations than was proliferation. Moreover, the antiproliferative effect of paclitaxel was greatly reduced when endothelial cell proliferation was assessed in treatment conditions similar to those used for the chemotaxis assay (4-h exposure to the drug), and no relevant antiproliferative effect was observed at the same doses that inhibited chemotaxis. These findings suggest that the effect of paclitaxel on endothelial cell motility might be more important than that on proliferation in the overall inhibition of angiogenesis *in vivo*. Paclitaxel might conceivably affect tumor-induced angiogenesis *in vivo* at local concentrations lower than those necessary to cause a cytotoxic effect on tumor cells. The cytotoxic properties of paclitaxel make it difficult to recognize whether its antineoplastic activity is due to inhibition of angiogenesis or to a direct toxic effect on tumor cells. More studies are thus needed to clarify the therapeutic relevance of these findings and to develop treatment strategies.

The second indication that the antiangiogenic effect of paclitaxel is not merely dependent on inhibition of proliferation is the lack of antiangiogenic activity of cisplatin, another cytotoxic antineoplastic drug. Cisplatin inhibited endothelial cell proliferation *in vitro* but not angiogenesis *in vivo* at doses that are related to the maximum tolerated dose and that have shown antineoplastic activity in murine systems (19, 20). This finding is in agreement with other studies describing the lack of antiangiogenic activity of cisplatin (21, 22). Both paclitaxel and cisplatin inhibited endothelial cell motility; however, in contrast to paclitaxel, the inhibition of cell motility by cisplatin appears to be a direct consequence of its high antiproliferative activity, since it occurred only at doses which inhibited cell proliferation.

Altogether, our findings indicate that the antiproliferative effect of paclitaxel is not the main mechanism for its antiangiogenic activity and that drugs which affect endothelial cell proliferation are not necessarily antiangiogenic. Our study also indicates that the *in vivo* Matrigel model of angiogenesis represents a good model to study true antiangiogenic drugs independently on their effect on endothelial cell growth.

The first evidence of an inhibitory activity of microtubule-affecting drugs on endothelial cell migration was reported in studies on an *in vitro* wound-healing assay using paclitaxel, colchicine, and vinblastine (2, 3, 23). Interestingly, in this assay, colchicine and vinblastine inhibited cell motility without affecting proliferation however (2). Recently, the inhibitory activity of paclitaxel on angiogenesis in the chick chorioallantoic membrane model has been reported, although no mechanism for this inhibition has been studied (24).

Paclitaxel also prevented two other functions related to angiogenesis involving the interaction of endothelial cells with the extracellular matrix: cord formation in a three-dimensional matrix and endothelial cell invasiveness through a layer of Matrigel. These processes require both motility and the production of matrix-degrading enzymes by invading cells. Paclitaxel affected both activities: besides its effect on motility, it also reduced the production of the matrix metalloproteinase MMP-2 by endothelial cells, as already reported for other cell types (8).

Antiangiogenic factors can act by different mechanisms: they can either act directly on endothelial cell functions or block angiogenesis indirectly, *e.g.*, by blocking the production of angiogenesis stimulating factors or by inhibiting the recruitment and activation of inflammatory cells known to release angiogenic factors (1, 16). The finding that paclitaxel inhibited several endothelial cell functions *in vitro* indicates that its antiangiogenic activity *in vivo* is not indirect, but that it acts directly on the endothelial cell response to angiogenic factors.

This study demonstrated that paclitaxel is a potent antiangiogenic agent. The effect does not appear to be a mere consequence of its cytotoxicity, since it inhibits other endothelial cell functions with even greater potency. We propose that the anti-angiogenic property of paclitaxel might contribute to its antineoplastic activity.

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EXHIBIT 2

[CANCER RESEARCH 62, 6938–6943, December 1, 2002]

Protracted Low-Dose Effects on Human Endothelial Cell Proliferation and Survival *in Vitro* Reveal a Selective Antiangiogenic Window for Various Chemotherapeutic Drugs[1]

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ABSTRACT

Recent preclinical studies have shown that frequent administration *in vivo* of low doses of chemotherapeutic drugs ("metronomic" dosing) can affect tumor endothelium and inhibit tumor angiogenesis, redueing significaunt side effeets (*e.g.*, myelosuppression) involving other tissues, even after chronic treatment. This suggests that aetivated endothelial cells may be more sensitive, or even selectively sensitive, to protraeted ("high-time") low-dose chemotherapy eompared with other types of normal cells, thus creating a potential therapeutic window. To examine this hypothesis, we assessed the effeets of several different chemotherapeutic drugs—namely paelitaxel, 4-hydroperoxyeyclophosphamide, BMS-275183 (an oral taxane), doxorubicin, epothilone B (EpoB) and its analogue 5-methylpyridine EpoB—on human microvaseular or maerovaseular endothelial cells, fibroblasts, and drug-sensitive or multidrug-resistant breast eaneer cell lines in cell culture, using both short-term (24 h) *versus* long-term (144 h), continuous exposures, where drug-containing medium was replaeed every 24 h. Whereas little differential and only weak effeets were observed using the short-term exposure, a striking trend of comparative vascular endothelial cell hypersensitivity was indueed using the continuous long-term exposure protoeol. Potent differential growth inhibition effeets as well as induetion of apoptosis were observed with IC_{50} values in the range of 25–143 pM for paelitaxel, BMS-275183, EpoB, and 5-methylpyridine-EpoB. In contrast, the IC_{50} values for tumor cells and fibroblasts tested were in the range of 500 pM to >1 mM for these drugs. Similar differential IC_{50} values were noted using 4-hydroperoxyeyelophosphamide. The results are eonsistent with the possibility that eontinuous low-dose therapy with various chemotherapeutie drugs may have a highly selective effeet against cycling vascular endothelial cells, and may be relevant to the use of eontinuous or frequent administration of low doses of eertain types of drugs as an optimal way of delivering antiangiogenie therapy.

INTRODUCTION

Among the many drugs capable of inhibiting angiogenesis in various preclinical models are conventional cytotoxic chemotherapeutic drugs (1). The first report of the antiangiogenic properties of such drugs appeared more than 15 years ago (2), and since then most drugs belonging to virtually every class of anticancer chemotherapeutic agents have been shown to have antiangiogenic effects (1). The basis for these effects, at least in part, is presumably related to the presence of cycling endothelial cells in newly forming blood vessel capillaries (3). Intcrest in cxploiting chemotherapeutic drugs for their antiangiogenic properties in the context of cancer therapy was stimulated recently by two reports (4, 5) using frequent administration of drugs such as cyclophosphamide or vinblastine *in vivo* at doses much lower than the MTD.[3] The rationale for this approach—called antiangiogenic or metronomic "chemotherapy" (4, 6)—is that damage to the tumor's vasculature using conventional regimens can be largely repaired during the long breaks (*e.g.*, 3 weeks) between successive cycles of MTD chemotherapy. Such breaks are normally required to allow for recovery from some of the harmful side effects of such therapies, such as myelosuppression (4). Shortening the time between cycles minimizes the efficacy of the repair process, which requires the use of lower doses of drug. Whereas this strategy seems to enhance the efficacy of the antiangiogenic effects of chemotherapy, in theory it should enhance the undesirable side effects of chemotherapy as well, including myelosuppression. Interestingly, this does not seem to be the case (4, 5, 7, 8) and implies that activated vascular endothelial cells may be more sensitive to certain low, or lower doses, of various chemotherapeutic drugs compared with other types of normal cells, or cancer cells, when exposed in a frequent or continuous manner.

Some preliminary evidence for such a preferential sensitivity of endothelial cells has been suggested on the basis of certain *in vitro* experiments. Thus, several groups have reported that very low (*e.g.*, nanomolar) concentrations of certain drugs such as PTX (7, 9), topotecan, campthothecin (10), or vinblastine (5, 7, 11) can significantly block endothelial cell growth, but not necessarily tumor cell growth *in vitro*. In addition, Vacca *et al.* (11) found that nanomolar or evcn picomolar concentrations of vinblastine, which did not causc significant antiproliferative effects against endothelial cells, could nevertheless suppress angiogenesis and certain endothelial cell functions considered relevant to angiogenesis, such as migration and protease production. Differential antiproliferative effects of chemotherapeutic drugs on vascular endothelial cells *versus* tumor cells have been noted with vinblastine, PTX, cisplatinum, and adriamycin (7).

All of the aforementioned *in vitro* experiments usually involved a single drug exposure of between 24 and 72 h. However, this does not mirror the *in vivo* situation when protracted low-dose metronomic chemotherapy protocols are administered frequently (*e.g.*, weekly; Ref. 4) every 3 days (5), or even daily (8, 12). We, therefore, asked whether protracted exposures *in vitro* to certain chemotherapeutic drugs might reveal new and important aspects of the antiendothelial cell effects of low-dose metronomic chemotherapy regimens. The purpose of this study was to examine this question and, more specifically, to determine whether there is an "antiangiogenic window" for antineoplastic chemotherapeutic drugs in which drug activity, at comparatively low concentrations, is largely or specifically restricted to endothelial cells. To do so, we designed long-term *in vitro* assays in which human tumor cells, dermal fibroblasts, and macrovascular or microvascular endothelial cells were exposcd daily, for up to 6 days,

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[3] The abbreviations usecl are: MTD, maximum tolcrated dose; EpoB, cpothilone B; 5-MP-EpoB, 5-mcthylpyridine EpoB; 4-HC, 4-hydropcroxycyclophosphamide; DXR, doxorubin; PTX, paclitaxel; HUVEC, human umbilical vein endothelial cell(s); HMVEC-d, human dermal microvascular endothelial cell(s); NHDF, normal human dermal fibroblast: VEGF, vascular endothelial growth factor; FBS, fetal bovine serum.

to various low concentrations of different, well known and novel chemotherapeutic drugs (Fig. 1), including 4-HC, PTX, an oral active taxane called BMS-275183 (13), DXR, EpoB (14), and the analogue 5-MP-EpoB (15). Our results show that vascular endothelial cells are, indeed, preferentially affected for properties such as proliferation and induction of apoptosis, by low concentrations of most of these drugs when exposed continuously for protracted periods of time (*e.g.*, 6 days), but not for shorter periods of time (*e.g.*, 24 h).

MATERIALS AND METHODS

Drugs. BMS-275183 (Bristol Myers-Squibb Co.) was provided by Drs. R. Malik and W. Rose. EpoB and 5-MP-EpoB were synthesized by the Nicolaou group (14, 16). 4-HC was a gift from Dr. M. Colvin (Duke University, Durham, NC). DXR and PTX were purchased from Sigma Chemical Co. To perform *in vitro* studies the compounds were stocked in 10 mM solutions: BMS-275183, EpoB, 5-MP-EpoB, and PTX were dissolved in 100% DMSO; and DXR was dissolved in distilled water. 4-HC was stocked as a powder at −20°C and dissolved for every treatment at 4°C in distilled water. All of the drugs were diluted in culture medium immediately before their use.

Cells and Culture Conditions. HUVEC, HMVEC-d, and NHDFs were purchased from Clonetics. The human breast cancer cell line MDA-MB-435 was obtained from Dr. J. Lemontt (Genzyme Corp., Cambridge, MA); and its P-glycoprotein-positive multidrug-resistant variant of MDA-MB-435, called T0.1, was obtained from Dr. D. Cohen (Novartis) and was derived by *in vitro* exposure to increasing concentrations of PTX.

The HUVEC and HMVEC-d cells were maintained in MCDB131 culture medium (JRH Biosciences) supplemented with 10% heat-inactivated FBS (Life Technologies, Inc.), L-glutamine (2 mM, Life Technologies, Inc.), heparin (10 units/ml; Wyeth-Ayerst), epidermal growth factor (10 ng/ml; Upstate Biotechnology Inc.), and basic fibroblast growth factor (5 ng/ml; R&D Systems, Inc.). Human endothelial cells were routinely grown in 1% gelatin-coated tissue culture flasks (Nunc A/S). NHDFs were cultured in 5% FBS RPMI medium (Life Technologies, Inc.) supplemented with L-glutamine; the breast cancer cell lines were maintained and expanded as monolayer culture in 10% FBS DMEM (Life Technologies, Inc.) supplemented with L-glutamine; the T0.1-resistant variant was grown with the addition of 0.1 μM PTX. Cells were kept in a humidified atmosphere of 5% CO_2 at 37°C and harvested with a solution of 0.25% trypsin-0.03% EDTA (Life Technologies, Inc.) when they were in log phase of growth, and maintained at the above-described culture conditions.

Cell Proliferation Assay. *In vitro* chemosensitivity testing was performed on single-cell suspensions of HUVEC, HMVEC-d, NHDF, MDA-MB-435, and T0.1 cells plated in 96-well plastic plates (1% gelatin coated for the endothelial cells) and allowed to attach overnight. Each drug concentration was represented by at least 10 wells and replicated three times. Cells were treated for 24 h (3×10^3 cells/well in 200 μl of medium) or 144 h (1×10^3 and 0.5×10^3 cells/well of normal and cancer cells, respectively, in 200 μl of medium) with PTX (1–1,000 pM), BMS-275183 (1–1,000 pM), EpoB (1–1,000 pM), 5-MP-EpoB (1–1,000 pM), 4-HC (0.1–10,000 nM), and DXR (1–1,000 nM) (Fig. 1); to maintain a constant concentration of the drugs during the protracted 144-h period of the experiments, every 24 h the medium was removed and fresh solutions were added with new medium (Fig. 1). The 4-HC treatments were performed as recommended by Flowers *et al.* (17) to avoid the toxic effects of volatile metabolites. At the end of the experiment, cells were pulsed for 6 h with 2 μCi/well of methyl-[^{3}H]-thymidine (Amersham Life Science), as described previously by Klement *et al.* (5). The concentration of drugs that reduced cell proliferation by 50% (IC_{50}) as compared with controls was calculated by nonlinear regression fit of the mean values of the data obtained in triplicate experiments.

Apoptosis Measurements. HUVEC, HMVEC-d, MDA-MB-435, T0.1, and NHDF cells were plated in 100-mm sterile dishes and continuously treated for 144 h, as described above, with 100 pM PTX, 100 pM BMS-275183, 100 pM EpoB, 100 pM 5-MP-EpoB, 100 nM 4-HC, and vehicle alone. At the end of the sixth day of treatment, cells were collected and the Cell Death Detection ELISA Plus kit (Roche) was used to quantify apoptosis as described previously (18) All of the absorbance values (measurement wavelength, 405 nm; reference wavelength, 490 nm) were plotted as a percentage of apoptosis relative to control cells (vehicle only), which were labeled as 100%. All experiments were repeated two times with at least two replicates/sample.

Statistical Analysis. The results (mean values ± SE) of cell proliferation, adhesion, and migration assays were subjected to statistical analysis by ANOVA, followed by the Student-Newman-Keuls test, using the GraphPad Prism© software package (version 3.0; GraphPad Software Inc.). The level of significance was set at $P < 0.05$.

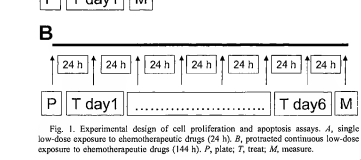

Fig. 1. Experimental design of cell proliferation and apoptosis assays. *A*, single low-dose exposure to chemotherapeutic drugs (24 h). *B*, protracted continuous low-dose exposure to chemotherapeutic drugs (144 h). *P*, plate; *T*, treat; *M*, measure.

RESULTS

Protracted Low-Dose Treatment with Taxanes, EpoB, and 4-HC Specifically or Preferentially Inhibits Endothelial Cell Proliferation. When endothelial cells (both macrovascular umbilical vein and dermal microvascular) were exposed to the low-dose schedule for only 24 h, significant inhibition was only observed at the highest concentrations of drug tested (*e.g.*, BMS-275183 46.9 ± 4.5%, and EpoB 33.4 ± 2.2% *versus* HUVEC control proliferation, $P < 0.05$; Fig. 2, *left*). In striking contrast, a strong and dose-dependent antiproliferative effect (including at the lower concentrations) was observed when endothelial cells were continuously exposed over 144 h (Fig. 2, *right*). PTX affected only endothelial cells from concentrations of 10 pM (77.3 ± 12% *versus* HMVEC-d control proliferation, $P < 0.05$; Fig. 2*A*, *right*) to 1 nM, which showed maximum inhibition (HUVEC 14.7 ± 2.1%, HMVEC-d 9.6 ± 3.5% *versus* control proliferation, $P < 0.05$; Fig. 2*A*, *right*), with a calculated IC_{50} of 51 and 96 pM for HMVEC-d and HUVEC, respectively. At the same low concentration, both breast cancer cell lines (parental and drug-resistant variant) showed no significant response, whereas the normal dermal fibroblasts were affected at 1 nM. This same trend was observed with the oral taxane BMS-275183 (Fig. 2*B*, *right*) with a maximum inhibition of endothelial cell growth at 1 nM (HUVEC 6.3 ± 1.3%, HMVEC-d 4.5 ± 2.3% *versus* control proliferation, $P < 0.05$; Fig. 2*B*, *right*) with an IC_{50} of 143 and 102 pM for HUVEC and HMVEC-d, respectively. In this case, there was the exception of a significant effect at the highest dose tested on the parental cancer cell line MDA-MB-435 (7.2 ± 1.5% *versus* control proliferation, $P < 0.05$, IC_{50} = 498 pM; Fig. 2*B*, *right*) but not the multidrug-resistant T0.1 variant. Thus, a relative therapeutic antiangiogenic window appeared well defined in this case, particularly in comparison with the PTX-resistant breast cancer cell line variant. EpoB, on the other hand, showed a potent antiproliferative activity at 1 nM after 144 h (Fig. 2*C*, *right*) on all cell lines. In this case, the range of endothelial specificity appears narrow compared with MDA-MB-435 (IC_{50} = 44 and 25 pM for HUVEC and HMVEC-d, respectively;

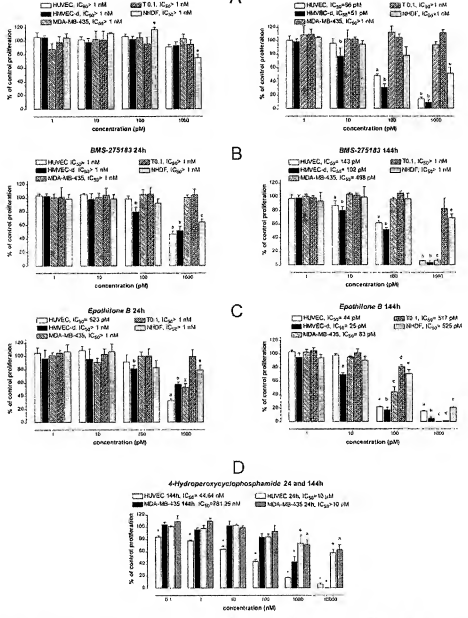

Fig. 2. Effect of low-dose PTX (*A*), BMS-275183 (*B*), EpoB (*C*), and 4-HC (*D*) on *in vitro* cell proliferation. The antiproliferative effects of the drugs were studied using both short-term (24 h, *left*) and prolonged continuous exposures (144 h, *right*) on the HUVEC, HMVEC-d, MDA-MB-435, T0.1, and NHDF cell lines. *Columns* and *bars*, mean values ± SE, respectively. $^*P < 0.05$ *versus* HUVEC controls; $^{\S}P < 0.05$ *versus* HMVEC-d controls; $^{\S}P < 0.05$ *versus* MDA-MB-435 controls; $^{\#}P < 0.05$ *versus* T0.1 controls; $^{\S}P < 0.05$ *versus* NHDF controls; $^{+}P < 0.05$ *versus* 144 h HUVEC controls; $^{\S}P < 0.05$ *versus* 144 h MDA-MB-435 controls; $^{-}P < 0.05$ *versus* 24 h HUVEC controls; $^{\S}P < 0.05$ *versus* 24 h MDA-MB-435 controls.

IC_{50} = 83 for MDA-MB-435), but not if compared with T0.1 (IC_{50} = 517 pM); in this case, the difference remained quite significant. In contrast, for all of the compounds tested, the normal human dermal fibroblasts were affected in their proliferation only at the highest concentrations used but never at the levels observed to be effective against endothelial cells (Fig. 2, *right*).

The toxic metabolite/alkylating agent 4-HC, a compound that in solution spontaneously reduces to 4-hydroxycyclophosphamide (the first active metabolite formed in the metabolism of cyclophosphamide), showed a significant dose-dependent inhibition of HUVEC proliferation (Fig. 2*D*, $P < 0.05$). This effect was observed at concentrations that did not affect MDA-MB-435 cancer cell growth. The IC_{50} in the 144-h treatments were 44.64 and 781.25 nM for HUVEC and the MDA-MB-435 cell line, respectively. As shown in Fig. 2*D*, the 4-HC concentrations targeting only endothelial cells were between 1 and 100 nM (*e.g.*, at 1 nM 77.7 ± 1% *versus* control proliferation, at 100 nM 43.7 ± 3.5% *versus* control proliferation; $P < 0.05$), which were in sharp contrast to the MDA-MB-435 breast cancer cell line (at 1 nM 95.6 ± 3.4% *versus* control proliferation, at 100 nM 83.7 ± 5.5% *versus* control proliferation).

Not All Antineoplastic Drugs Have a Specific Antiproliferative Activity Against Endothelial Cells. In contrast to the above mentioned compounds, DXR and 5-MP-EpoB did not show a significant difference in their relative effects on the endothelial and the cancer cell

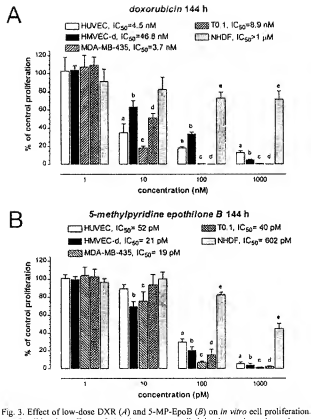

Fig. 3. Effect of low-dose DXR (*A*) and 5-MP-EpoB (*B*) on *in vitro* cell proliferation. The antiproliferative effects of the drugs were studied in the prolonged continuous exposure of 144 h on the HUVEC, HMVEC-d, MDA-MB-435, T0.1 and NHDF cell lines. *Columns* and *bars*, mean values ± SE, respectively. $^{a}P < 0.05$ *versus* HUVEC controls; $^{b}P < 0.05$ *versus* HMVEC-d controls; $^{c}P < 0.05$ *versus* MDA-MB-435 controls; $^{d}P < 0.05$ *versus* T0.1 controls; $^{e}P < 0.05$ *versus* NHDF controls.

lines (Fig. 3, *A* and *B*, respectively) using the low-dose protracted time course regimen. The degree of the antiproliferative activity was the same against endothelial cells and cancer cells (*e.g.*, DXR IC_{50} = 4.5 nM and 3.7 nM for HUVEC and MDA-MB-435, respectively; or 5-MP-EpoB IC_{50} = 21 pM and 19 pM for HMVEC-d and MDA-MB-435, respectively), suggesting that these drugs might act as antitumor compounds by affecting both cancer and endothelial cells at the same drug concentration. These results also show that the selectivity of endothelial cells to the other drugs we tested is not simply a function of decreased "hardiness" of such cells, compared with fibroblasts or tumor cells.

Protracted Low-Dose Chemotherapy *in Vitro* Induces Endothelial Cell Apoptosis. To further investigate the specific effect of treatments on endothelial cells, an ELISA assay was performed to quantify the cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) after induced cell death in treated samples. As shown in Fig. 4, the assay revealed a markedly higher increase of apoptosis in the treated endothelial cells when compared with the cancer cell lines or fibroblasts, after the 6 days of treatment, especially with 100 pM PTX (+210% and +230% for HUVEC and HMVEC-d *versus* controls, respectively) and 100 nM 4-HC (+223% and +211% for HUVEC and HMVEC-d *versus* controls, respectively). In these experiments, 5-MP-EpoB did not show any selective effect on endothelial cells.

DISCUSSION

Our results highlight the importance of the concept of "high-time" chemotherapy (19, 20), *i.e.*, that prolonged exposure times, once an effective dose of drug is achieved (which can be quite low), is the critical parameter in cell kill, and may even afford the attainment of selectivity. High-time chemotherapy has been discussed previously from the perspective of tumor cell kill (19, 20), but our results suggest that it may also apply to endothelial cell kill and inhibition of growth as well.

Long-term, continuous-like exposure of cells in culture to anticancer chemotherapeutic drugs has not been undertaken previously because the main objective of such experiments in the past has been to study the toxic effects of relatively high concentrations of such drugs on tumor cells. Therefore, brief exposure (*e.g.*, 1 h), followed by assessment of effects on tumor cell viability and clonogenicity, have been typical in such experiments. The resulting IC_{50} values of conventional chemotherapeutic agents usually depended on numerous factors such as exposure time, pharmaceutical preparation, drug metabolism, and the type of cancer cells treated. Because of such factors, especially the short exposure times, the published effective IC_{50} drug concentrations on human cells of drugs such as PTX (21), EpoB (21), and BMS-275183 (13) tend to be much higher than the results of our experiments. In contrast, we studied the effects of protracted drug exposure using very low concentrations of drug and tested the effects of such protocols not only on tumor cells but on normal fibroblasts and vascular endothelial cells. The rationale for doing so stems from the concept of using continuous low-dose metronomic/antiangiogenic chemotherapy regimens as a means of inducing antitumor effects *in vivo* secondary to an effect on the endothelial cells of a tumor's vasculature (4, 5). Results by Browder *et al.* (4) and Klement *et al.* (5) have shown this can cause long-term growth control of transplanted tumors with little host toxicity, and without the rapid development of drug resistance. Indeed, tumors selected for high levels of acquired resistance can be induced to respond by using metronomic/antiangiogenic chemotherapy regimens (4, 7), especially when they are combined with an antiangiogenic drug such as TNP-470 or anti-VEGF receptor-2 blocking antibodies (4, 7).

Our results are highly suggestive of an antiangiogenic window when low-dose chemotherapy is used, which becomes apparent after protracted exposure times (*e.g.*, 144 h) where drug-containing medium is replaced on a daily basis. There was a clear trend showing the effectiveness of low concentrations of drug that had no or little effect at low dose on tumor cells or normal fibroblasts (*e.g.*, 25–150 pM for PTX, BM-S275183, or EpoB), in contrast to microvascular endothelial cells. This was the case not only for inhibition of proliferation but also for the induction of apoptosis. Other functions such as cell migration and adhesion to extracellular matrix, which are all consid-

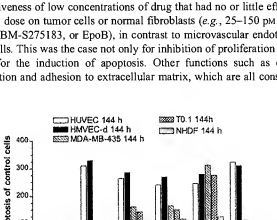

Fig. 4. Induction of cell apoptosis after prolonged (144 h) low-dose treatment with chemotherapeutic drugs on the HUVEC, HMVEC-d, MDA-MB-435, T0.1 and NHDF cell lines. Apoptosis was detected by a Cell Death ELISA kit, and the results were plotted as a percentage of the apoptosis of control cells (100%).

ered relevant to angiogenesis, are also similarly affected in a differential and selective manner (data not shown).[4]

Taken together, the results provide further evidence that some of the antitumor effects of anticancer chemotherapy drugs may be attributable to inhibition of tumor angiogenesis (1). Moreover, it may be possible to significantly lower drug concentrations with the aim of reducing overall host toxicity, but without sacrificing and even increasing antitumor efficacy, as emphasized by Browder *et al.* (4), Klement *et al.* (7), and Bello *et al.* (22). An additional benefit would be to delay acquired drug resistance (5), and even to treat tumors that are already resistant to the very drugs used for the low-dose chemotherapy (5, 7), given the relative genetic stability of normal host vascular endothelial cells, in contrast to tumor cells (23).

Whereas our experiments have dealt mainly with conventional or new cytotoxic chemotherapy drugs, the results may apply to other types of anticancer drugs as well. Indeed, we have found that BAL-9504, a geranylgeranyl-transferase inhibitor (24), had selective anti-endothelial cell effects using protracted low-dose exposures.[4] We have been studying BAL-9504 in our analysis because there is evidence that similar drugs (*e.g.*, Ras farnesyltransferase inhibitors) may have direct endothelial cell effects and, hence, might inhibit angiogenesis through such effects (25) as well as by interfering with certain tumor cell functions relevant to angiogenesis, such as VEGF production (26).

Four other aspects of our experimental system, and results, need to be emphasized. First, the relative sensitivity of vascular endothelial cells to protracted low-dose chemotherapy may vary with the organ origin of endothelial cells. In addition, a potential weakness of our study is that we did not include types of normal cell that are ordinarily highly sensitive to chemotherapy *in vivo* (*e.g.*, gut mucosal epithelial cells, hair follicle cells, or bone marrow progenitors). It is possible that such cell types may show a high degree of sensitivity to protracted exposures of low concentrations of chemotherapeutic drugs, similar to endothelial cells. This is under investigation. Second, our previous *in vivo* results using regimens involving frequent or continuous administration of low-dose chemotherapy (5, 7, 12) have emphasized the critical need for combining such regimens with a second antiangiogenic agent, such as anti-VEGF receptor-2 blocking antibody (5, 7), a finding confirmed by others (22, 27–29). For example, the intrinsic elevated sensitivity of activated endothelial cells, to low-dose chemotherapy, compared with other cells, may not be expressed as a result of the presence of high local concentrations of endothelial cell-specific survival factors such as VEGF (30–33). Such combinations may be particularly effective in inducing higher levels of apoptosis of activated endothelial cells (7) coupled with the inhibition of cell proliferation, especially when used in a protracted manner. Third, an obvious question raised by our results is why vascular endothelial cells appear to be selectively sensitive to protracted exposure of low concentrations of chemotherapeutic drugs. It is possible that the inhibition of endothelial cell growth or induction of apoptosis may not be directly mediated by the chemotherapeutic drugs tested, but rather are secondary to an event induced by the drugs (*e.g.*, a change in expression of genes or proteins that mediate in some fashion the selective antiendothelial effects we observed). Fourth, it is interesting that some of the drugs we tested expressed potent antitumor cell activity as well as antiendothelial cell activity, when used at ultra-low concentrations in a protracted manner (*e.g.*, EpoB). Such an agent, if it functioned in a similar manner *in vivo*, may be particularly useful for metronomic chemotherapy protocols because it may affect both the tumor cell and endothelial compartments in a chronic manner, and do so in a way that avoids the acute toxicities normally associated with MTD-based, tumor cell-directed, chemotherapeutic regimens.

In summary, our results add to a small but important, and growing, body of literature implicating conventional (and new) chemotherapy drugs as antiangiogenics, a property that can be enhanced by protracted exposures to low doses of drug. Our results may also be directly relevant to *in vivo* therapy studies using other known, or potential, antiangiogenic agents such as IFNα and endostatin where these drugs appear to have greater antiangiogenic and antitumor effects when administered frequently or continuously at low doses (34–36).

[4] G. Bocci and R. S. Kerbel, unpublished observations.

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EXHIBIT 3

Paclitaxel at ultra low concentrations inhibits angiogenesis without affecting cellular microtubule assembly

Jieyi Wang[a], Pingping Lou[a], Rick Lesniewski[a] and Jack Henkin[a]

Many conventional chemotherapeutics, such as the microtubule-stabilizing anticancer drug paclitaxel (Taxol), have been shown to have anti-angiogenic activity and clinical application of a continuous low dose of these agents has been suggested for cancer therapy. In this study, we show that paclitaxel selectively inhibits the proliferation of human endothelial cells (ECs) at ultra low concentrations (0.1–100 pM), with an $IC_{50} = 0.1$ pM, while it inhibits non-endothelial type human cells at 10^4- to 10^5-fold higher concentrations, with $IC_{50} = 1–10$ nM. The selectivity of paclitaxel inhibition of cell proliferation is also species specific, as mouse ECs are not sensitive to paclitaxel at ultra low concentrations. They are inhibited by higher concentrations of paclitaxel with $IC_{50} = 1–10$ nM. Inhibition of human ECs by paclitaxel at ultra low concentrations does not affect the cellular microtubule structure, and the treated cells do not show G_2/M cell cycle arrest and apoptosis, suggesting a novel but as yet unidentified mechanism of action. In an *in vitro* angiogenesis assay, paclitaxel at ultra low concentrations blocks human ECs from forming sprouts and tubes in the three-dimensional fibrin matrix. In summary, paclitaxel selectively inhibits human EC proliferation and *in vitro* angiogenesis at low picomolar concentrations. The data support a clinical application of continuous ultra-low-dose paclitaxel to treat cancer. *Anti-Cancer Drugs* 14:13–19 © 2003 Lippincott Williams & Wilkins.

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Keywords: angiogenesis, endothelial cell, Taxol, paclitaxel

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Introduction

Angiogenesis, the formation of new blood vessels from existing vasculature, is an essential component of a variety of pathological states including tumor growth, diabetic retinopathy, macular degeneration, arthritis and inflammation [1,2]. Proliferation of normally quiescent endothelial cells (ECs) is one of the critical steps required for angiogenesis. Inhibition of endothelial proliferation has been shown to be effective in blocking angiogenesis and tumor growth in animal models [3,4]. Anticancer chemotherapeutic agents, while targeting cancer cells directly, have inhibitory effects on vascular ECs, which may have contributed to the anti-cancer efficacy of these agents [5]. In fact, anti-endothelial effects have been demonstrated for vinblastine, cyclophosphamide, 5-flurouracil (5-FU), paclitaxel, doxorubicin and others [6,7]. Furthermore, Browder *et al.* has reported that anti-angiogenic scheduling of chemotherapy improved efficacy against drug-resistant tumors in animal models [8]. The anti-angiogenic-dosing schedule was designed to administer chemotherapeutics at shorter intervals without interruption, to prevent recovery of ECs and, therefore, to achieve more effective suppression of the proliferating ECs in the tumor bed. Klement *et al.* showed that continuous low-dose vinblastine administration caused a direct anti-angiogenic effect *in vivo*, and that combination treatment of low-dose vinblastine with VEGF receptor-2 antibody resulted in full and sustained regressions of large established tumors without an ensuing increase in host toxicity or drug resistance [9]. These studies have demonstrated the potential usefulness of EC growth inhibitors in cancer treatment.

In an effort to identify more potent EC inhibitors, we tested many conventional chemotherapeutics in a proliferation assay of human microvascular ECs. Paclitaxel showed selective inhibitory activity in human ECs at ultra low concentrations (0.1–100 pM). We further show that human ECs are more sensitive than mouse ECs and that the mechanism of this activity of ultra low concentrations of paclitaxel is not through disrupting cellular microtubules. The data support the use of ultra low paclitaxel in treating cancers in the clinic.

Materials and methods

Paclitaxel was purchased from Sigma (St Louis, MO), and was dissolved in DMSO at 10 mM and stored frozen at −20°C in aliquots. Other reagents were commercially obtained in the highest quality available.

Cell culture and proliferation assay

All normal human primary cells and their recommended culture media were purchased from Clonetics (San Diego, CA) as described earlier [10]. These cells included

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human neonatal dermal microvascular ECs (HMVECs), human umbilical vein ECs (HUVECs), human umbilical artery ECs (HUAVECs), normal human astrocytes (NHAs), normal human dermal fibroblasts (NHDFs), normal human epidermal keratinocytes (NHEKs), human mammary epithelial cells (HMEpCs), human prostate epithelial cells (PrEpCs) and human umbilical artery smooth muscle cells (UASMCs). Cells were grown according to instructions from Clonetics, and cell proliferations were performed in 96-well plates using cells between passages 6 and 12. Cells were seeded at 3000–5000 cells/well and allowed to attach for 4 h. Paclitaxel and other agents, diluted in culture medium, were added in quadruplicate wells and the cells were incubated for 3 days before MTS reagents (Promega, Madison, WI) were added to quantitate the live cells in each well.

Transformed HUVECs were prepared in order to extend the *in vitro* proliferative lifespan of primary HUVECs (Clonetics); the cells were transformed using SV40 Tag [11]. Briefly, the HUVECs were infected (m.o.i. = 1) with a recombinant, defective retrovirus transducing the SV40 TAg and *hygB* genes. Clonal cell lines were obtained from the hygromycin-resistant cell populations. Flow cytometric analysis indicated that tumor necrosis factor-α-induced expression of E-selectin, ICAM-1 and VCAM was similar in both the primary and Tag-transformed HUVECs.

Primary mouse brain microvascular ECs (MBMECs) and growth medium were purchased from Cell Applications (San Diego, CA). A mouse EC line derived from brain microvasculature (BEND3) was kindly provided by W. Risau [12]. SVEC4-10, a mouse EC line derived by SV40 transformation of ECs from auxiliary lymph node vessels, was purchased from ATCC (Rockville, MD). Both BEND3 and SVC4-10 cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS). Proliferation assays were performed in a similar fashion as described above.

Cell cycle analysis by flow cytometry

HMVECs were grown in EGM2 media (containing growth factors VEGF, FGF, EGF and IGF, and 5% FBS) in T75 culture flasks at 50–60% confluency when paclitaxel was added for final concentration of 0 or 0.1 pM to 10 μM. The cells were incubated at 37°C and 5% CO_2 for 48 h. Cell morphology was recorded, and then cells were detached by trypsinization and stained with propidium iodide for flow cytometric analysis. Briefly, cells were washed with PBS twice and fixed with 70% ethanol. Cell pellets were then resuspended in PBS containing 0.6% NP-40, 10 mg/ml RNase and 0.1 mg/ml propidium iodide. The cells were analyzed on a FACS-Calibur instrument (Becton Dickson, San Jose, CA).

Cellular microtubule staining

HMVECs were grown on eight-well chamber slides at 20 000 cells/well in EGM2 media. The cells were incubated with 0 or 0.1 pM to 1 μM paclitaxel for 48 h. The cells were fixed with 10% formalin and stained with a rat anti-tubulin IgG followed by FITC-conjugated goat anti-rat antibody (Chemicon, Temecula, CA). Cellular microtubule structures were viewed in a confocal fluorescence microscope and digital images were taken.

EC tube formation in the fibrin matrix

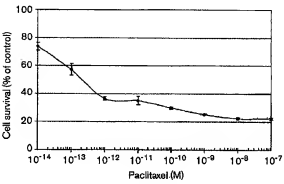
This protocol was adapted from published methods [13]. HMVECs were mixed with gelatin-coated Cytodex microcarrier beads (Sigma) at 1×10^6 cells/ml and 30% (v/v) beads in EGM2 media (containing growth factors supplements and 5% FBS). The cells/beads was incubated at 37°C/5% CO_2 for 4 h with gentle mixing every 30 min. Then, 10-fold more EGM2 was added and the mix was incubated overnight at 37°C/5% CO_2. Cells were normally at confluency on the bead surface after the incubation. The cells/beads were resuspended in fresh EGM2 media at 1% (v/v) and mixed with an equal volume of 6 mg/ml human fibrinogen (Sigma) in EBM2 basic medium. Human thrombin (Sigma) was then added to give a final concentration of 0.05 U/ml and the mixture was dispersed to a 24-well plate, 1 ml/well. The fibrin gel formed within 4 min at room temperature and 1 ml of EGM2 media with 0 or 0.1 pM to 10 μM paclitaxel was then added into the wells with HMVECs/beads embedded in the fibrin matrix. The plate was incubated at 37°C/5% CO_2 for 72 h, and tube formation was checked under phase-contrast microscopy and digital images were taken. Similar experiments were carried out with BEND3 cells.

Results

Paclitaxel inhibits EC proliferation at ultra low concentrations

Several widely used cancer chemotherapeutics were tested for their effects on human EC proliferation, among which paclitaxel showed exceptional potency against the growth of HMVECs. Paclitaxel showed an inhibitory effect on HMVEC proliferation at ultra low concentrations (0.1–100 pM) with an $IC_{50} = 0.1$ pM (Fig. 1 and Table 1). 5-FU, camptothecin, cisplatin and doxorubicin were active against HMVEC proliferation, but their effect is at higher concentrations in the nanomolar range (Table 1), not drastically different from their activity against other cells as reported [14]. The anti-proliferative activity of paclitaxel at ultra low concentrations on HMVECs prompted us to study its activity against primary cultures of a variety of cell types.

Fig. 1



Inhibition of HMVEC proliferation by paclitaxel. HMVECs grown in 96-well plates with complete growth medium EGM2 containing FBS and growth factors were exposed to paclitaxel at various concentrations as shown. The cells were allowed to grow for 3 days in the presence of the drug. Total live cells in each well at the end of incubation were quantified with MTS reagents. Data shown are from one typical experiment which were confirmed by two independent tests. Paclitaxel showed potent inhibition of HMVEC proliferation even at ultra low concentrations (0.1–100 pM).

Table 1 **Inhibition of HMVEC proliferation by chemotherapeutics**

Compounds	IC_{50} (pM)
Paclitaxel	0.1
5-FU	5000
Camptothecin	10000
Cisplatin	5000000
Doxorubicin	100000

HMVEC were grown in a 96-well plate with EGM2 medium containing growth factors and FBS. The cells were incubated with compounds at various concentrations for 3 days before MTS reagents were used to quantify the live cells. The IC_{50} (concentration to achieve 50% of maximum inhibition) was the average of three independent experiments.

Cell growth inhibition by paclitaxel at ultra low concentrations is selective for EC type

Human ECs of other origins and non-EC types of primary cells were tested against paclitaxel in 3-day proliferation assays. The IC_{50} of paclitaxel inhibition of growth of these cells is listed in Table 2. All human ECs, including HMVECs, HUVECs and HUAECs, were inhibited by ultra low concentrations of paclitaxel with an IC_{50} of 0.1 pM. Transformed HUVECs were similarly sensitive to the ultra low paclitaxel. However, non-EC types of human cells, including NHAs, NHDFs, NHEKs, HMEpCs, PrEpCs and UASMCs, were all significantly less sensitive to the inhibition of paclitaxel. The IC_{50} values for these cells were near 1 nM, which was in line with the anti-mitotic activity of paclitaxel. It was striking that proliferation of non-EC cell types was 6000- to 55 000-fold less sensitive to paclitaxel compared to human EC types tested. Selective inhibition of EC proliferation is one of the approaches to inhibit angiogenesis. The high

Table 2 **Inhibition of proliferation of human primary cells by paclitaxel**

Cell types	IC_{50} (pM)
HMVEC	0.1
HUVEC	0.1
HUAEC	0.1
NHA (astrocytes)	5500
NHDF (fibroblasts)	700
MepC (mammary epithelial cells)	1000
PrEpC (prostate epithelial cells)	650
UASMC (smooth muscle cells)	1000

Cells were grown and proliferations were performed as described in Materials and methods. The cells were incubated with compounds at various concentrations for 3 days before MTS reagents were used to quantify the live cells. The IC_{50} (concentration to achieve 50% of maximum inhibition) was the average of three independent experiments.

Table 3 **Inhibition of proliferation of EC of various species by paclitaxel**

EC types	IC_{50} (pM)
Human	
HMVEC	0.1
HUVEC	0.1
HUAEC	0.1
HUVEC (transformed)	0.1
Mouse	
BEND3	1000
SVEC4-10	10000
MBMEC	900

Cells were grown and proliferations were performed as described in Materials and methods. The cells were incubated with compounds at various concentrations for 3 days before MTS reagents were used to quantify the live cells. The IC_{50} (concentration to achieve 50% of maximum inhibition) was the average of three independent experiments.

potency and selectivity of paclitaxel against ECs makes it an exceptional agent for anti-angiogenic therapy.

Mouse ECs show responses only to higher concentrations of paclitaxel

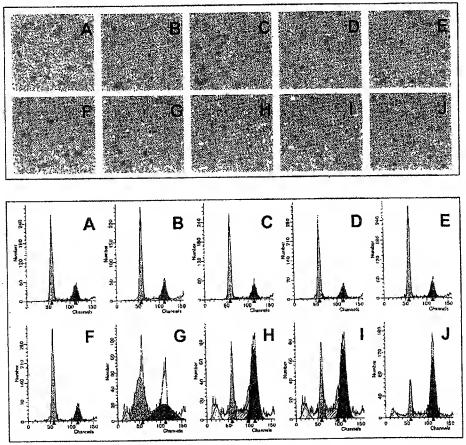
It is widely appreciated that ECs from different species have significant differences in response to angiogenesis inhibitors. We were interested in the sensitivity of mouse ECs to paclitaxel as anti-angiogenic agents would be tested in mouse models before going to the clinic. Three sources of mouse ECs were tested—BEND3 was an immortalized mouse brain capillary EC line [12], SVEC4-10 was an EC line derived by SV40 transformation of ECs from mouse auxiliary lymph node vessels and MBMECs were primary mouse brain microvascular ECs derived from normal mouse brain tissues. Proliferation of these mouse ECs was inhibited by paclitaxel at higher concentrations (Table 3), sensitivity being much lower as compared to that of human ECs. The primary mouse ECs (MBMECs) were inhibited with $IC_{50} = 0.3$ nM, similar to that for human non-EC primary cells. The immortalized mouse ECs, BEND3 and SVEC4-10, were even less responsive to the inhibition of paclitaxel. The lack of sensitivity of mouse ECs to ultra low concentrations of paclitaxel makes it less attractive to test the anti-angiogenic activity of ultra low paclitaxel in mouse angiogenesis and tumor models.

Paclitaxel at ultra low concentrations does not arrest HMVECs at the G_2/M phase of the cell cycle and does not affect cellular microtubules

To explore the mechanism of HMVEC growth inhibition by paclitaxel at ultra low concentrations, we studied cell morphology, cell cycle progression and cellular tubulin of HMVECs treated with paclitaxel. The cells treated with 0.1–1000 pM paclitaxel showed reduced cell density, consistent with the data as in the MTS quantification in Fig. 1. The cell morphology did not appear different from that of untreated cells (Fig. 2, upper panel). Flow cytometry analysis of cell cycle distribution of HMVECs treated with paclitaxel showed G_1 arrest of cells exposed to ultra low drug concentrations (Fig. 2, lower panel). In contrast, paclitaxel at higher concentrations (above 1 nM) caused the cells to detach from the culture plate, and induced cell cycle arrest at the G_2/M phase and apoptosis, consequences of paclitaxel affecting microtubules.

The morphology and cell cycle analysis data indicated that cell growth inhibition by paclitaxel at ultra low concentrations was not through disrupting cell microtubules. To confirm this hypothesis, we performed microtubule staining in cells treated with various concentrations of paclitaxel. The fine cellular microtubule network in cells treated with 0.1–100 pM paclitaxel was similar to that of untreated cells (Fig. 3). However, paclitaxel at higher concentrations (above 1 nM) showed

Fig. 2

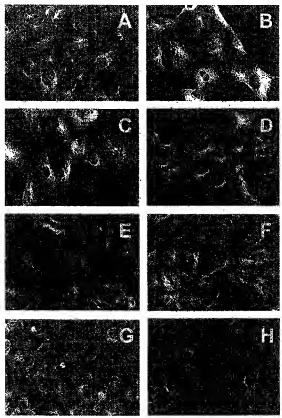


A = normal B = 0.1 pM C = 1 pM D= 10 pM E = 100 pM

F = 1 nM G = 10 nM H = 100 nM I = 1 μM J = 10 μM

Effect of paclitaxel on HMVEC morphology and the cell cycle. HMVECs were grown in T75 flasks with EGM2 medium with 5% FBS (A, normal) and with paclitaxel added at various final concentrations as shown (B–J). After 48 h incubation, cells treated with ultra low concentrations (0.1–100 pM) of paclitaxel showed lower cell density as compared to control (upper panel). These treated cells were all collected and subjected to flow cytometry analysis of DNA profiles (lower panel). Cells treated with paclitaxel concentrations above 1 nM (G–J) were mostly rounded up and not attached to flasks, and they were blocked at the G_2/M phase of the cell cycle and went to apoptosis.

Fig. 3



A = normal; B = 0.1 pM; C = 1 pM; D = 10 pM;
E = 100 pM; F = 1 nM; G = 10 nM; H = 1 μM

Effect of paclitaxel on HMVEC microtubules. HMVECs were grown in eight-well chamber slides in normal culture medium containing various concentrations of paclitaxel for 48 h. The cells were then fixed and stained for cellular tubulins. Cellular microtubules were observed with immunofluorescent microscopy. Paclitaxel at ultra low concentrations (0.1–100 pM, B–E) did not affect the cellular microtubule assembly.

the expected cellular effect, i.e. interference with microtubule assembly in HMVECs.

Paclitaxel at ultra low concentrations inhibits angiogenesis *in vitro*

To show paclitaxel at ultra low concentrations has anti-angiogenic activity, we tested it in an EC sprouting and tube formation angiogenesis model *in vitro*. HMVECs attached to microcarrier beads imbedded in fibrin matrix were able to sprout and form tubule structures (Fig. 4). This EC property was dependent on the presence of angiogenesis stimulators VEGF and bFGF. Paclitaxel at ultra low concentrations (0.1–100 pM) inhibited the HMVEC tube formation in this three-dimensional fibrin matrix. Mouse EC BEND3 were studied in a similar fashion, but paclitaxel did not show any inhibition at concentrations below 10 nM (data not shown). These data further show paclitaxel at ultra low concentrations is a specific inhibitor of human ECs.

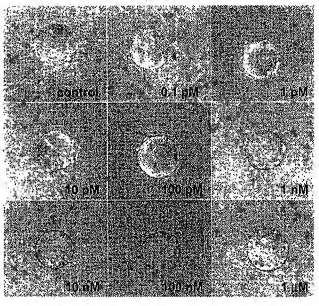
Discussion

Conventional chemotherapeutics inhibit tumor cell growth by blocking topoisomerase II activity (e.g. camptothecin), alkylating DNA (cisplatin), interfering with cellular microtubules (vinblastine and paclitaxel) or inhibiting metabolism (5-FU and methotrexate). The non-selective cell killing mechanism of action of these agents dictates they may inhibit EC growth as well and can be used as 'accidental' anti-angiogenic inhibitors when dosed appropriately. Application of chemotherapeutics for anti-angiogenesis cancer therapy was proposed in 1991 [15] and has been recently reviewed [2,7]. Recent studies have shown continuous low-dose application of chemotherapeutics has resulted in regression of large established tumors and inhibition of angiogenesis in animal models, without major toxicities or signs of acquired drug resistance during the course of treatment in mice [8,9]. Therefore, further analysis and evaluation of chemotherapeutics as antiangiogenic agents should uncover more benefits of these agents in cancer therapy.

Microtubule-interfering agents were among the first chemotherapeutics reported to have an anti-angiogenic effect [6]. In that report, Baguley *et al.* showed that vinblastine inhibited the growth of a drug-resistant colon adenocarcinoma and linked the efficacy to vascular collapse. Recently, the anti-angiogenic activity of paclitaxel has been described [16–18]. The angiogenesis in matrigel implants induced by tumor-derived stimulators was strongly inhibited in a mouse model with i.v. injections of 20–28 mg/kg paclitaxel at day 0 and 4 [16]. The dose was similar to conventional cytotoxic therapy regimen and not far away from the maximum tolerated dose of paclitaxel. In more recent studies, paclitaxel at 3–6 mg/kg daily dosing was found to be effective in inhibiting mouse cornea angiogenesis and intratumoral angiogenesis in a mouse model [17,18].

In the current investigation, we found that paclitaxel had direct effects on human ECs at ultra low concentrations (0.1–100 pM). Proliferation of human ECs was completely blocked by low picomolar concentrations of paclitaxel. This effect was seen in several commonly used human ECs, including HMVECs, HUVECs and HUAVECs. A direct effect of paclitaxel at a concentration as low as 10 pM on EC migration was reported before [16]. The potent EC inhibitory activities of paclitaxel were further demonstrated in our three-dimensional tube formation assay. Paclitaxel at 1 pM completely prevented HMVECs from sprouting and forming tubular structures in fibrin matrix, a process closely mimicking angiogenesis *in vivo* which requires ECs to migrate and proliferate.

Fig. 4



Inhibition of HMVEC sprouting and tube formation in the fibrin matrix. HMVECs grown on microcarrier beads sprouted and formed tubes when imbedded in fibrin gel with growth factors and FBS (EGM2 medium). Paclitaxel was able to completely suppress this endothelial function at ultra low concentrations, further indicating the anti-angiogenic activity of ultra-low-dose paclitaxel.

The lack of inhibition of mouse ECs by ultra low concentrations of paclitaxel is intriguing, and indicates it may not be possible to show *in vivo* efficacy of ultra low paclitaxel in mouse models. However, paclitaxel was active against mouse ECs at low nanomolar concentrations, so it is still one of the most potent among all anti-angiogenic agents. The efficacy of low-dose 6 mg/kg daily administration of paclitaxel inhibiting mouse cornea and tumor angiogenesis strongly supports the anti-angiogenic activity of paclitaxel. Human ECs are 10 000-fold more sensitive to the inhibition of paclitaxel than mouse ECs. This ultra sensitivity indicates that paclitaxel can be used at a much lower dose continuously in humans to achieve anti-angiogenic effects and that it may cause minimal toxicity considering many human non-EC cell types are not affected by ultra low concentrations of paclitaxel.

The mechanism of paclitaxel's anti-mitotic effect has been shown to be the inhibition of microtubule depolymerization and blockade of cell division in the G_2/M phase of the cell cycle [14]. Paclitaxel blocks mitosis and induces multi-nucleation of cells during interphase. These are associated with formation of an incomplete metaphase plate of chromosomes and an altered arrangement of spindle microtubules. These cellular effects of paclitaxel are achieved at low nanomolar drug concentrations in cell culture. Human ECs are inhibited by paclitaxel at ultra low concentrations (0.1–100 pM)—5000-fold lower than the concentrations needed for anti-mitotic effect. The mechanism of ultra low paclitaxel is not known. Our data suggest a novel mechanism of action that does not rely on disrupting microtubule assembly. First, inhibition of EC proliferation at pM concentrations of paclitaxel was cytostatic and no cell apoptosis was detected either by flow cytometry or by caspase-3 measurement (data not shown). The cells were arrested mostly at the G_1 phase of the cell cycle, whereas cells treated with higher concentrations of paclitaxel showed G_2/M arrest and apoptosis. Next, the morphology of human ECs treated with picomolar concentrations of paclitaxel was not different from untreated cells except for lower density, but cells exposed to higher paclitaxel concentrations became round and not well attached. Finally, cellular microtubule staining of human ECs treated with paclitaxel at concentrations below 1 nM did not show an abnormal microtubule network. Only in cells treated with higher concentrations was the condensed microtubule observed. Thus, the direct effect on the microtubule structure was not caused by ultra low concentrations of paclitaxel. Distinct sensitivity of ECs versus non-ECs to ultra-low-dose paclitaxel suggests the presence of specific targets in human ECs. We are currently investigating the mechanism at the molecular level, by examining alterations

in signal transduction in ECs treated with ultra low concentrations of paclitaxel.

'Metronomic' dosing, or anti-angiogenic scheduling, of cancer chemotherapeutics has been increasingly recognized to be a potential application of these agents in cancer therapy [2,7]. Data presented in this study, along with published observations [16–18], suggest that paclitaxel is a candidate of choice for clinical exploration. Paclitaxel at ultra low concentrations is not only potent at inhibiting angiogenesis, but also selective against ECs, as non-EC types are not inhibited by such ultra low concentrations of paclitaxel. Other chemotherapeutic agents, such as 5-FU, camptothecin and doxorubicin, are non-selective, and they inhibit ECs and non-ECs with similar IC_{50} (5–100 nM, Table 1 and data not shown). The unique properties of paclitaxel make it a powerful tool in cancer therapy. It can be envisaged that paclitaxel can be administered to maintain continuous drug concentrations of 100–1000 pM in plasma for long-term control of cancer following conventional treatment, such as surgery, radiation or chemotherapy. The suggested plasma drug concentrations of 100–1000 pM is higher than that needed for *in vitro* activity in consideration of the high plasma protein binding of this agent (89–98%) and paclitaxel at this concentration range still possesses cell-type selectivity. Clinical experience of paclitaxel accumulated over the last decade should guide us to deliver this agent to achieve the desired blood drug concentrations. Acquired drug resistance of continuous ultra-low-dose paclitaxel is not expected because ECs are the intended targets, which are considered genetically stable. Since normal non-EC types are not inhibited by paclitaxel at concentrations below 1000 pM, toxicity of ultra-low-dose paclitaxel is expected to be minimum. Angiogenesis-suppressed tumors are deprived of nutrients and growth and survival factors/signals from vessels and ECs, and are prone to apoptosis and more sensitive to radiation [19]. This continuous ultra-low-dose regimen may also be used in combination with radiation or surgery to enhance efficacy. Finally, combination of continuous ultra-low-dose paclitaxel with other novel angiogenesis inhibitors may also provide added clinical benefits.

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EXHIBIT 4

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Increased Endothelial Uptake of Paclitaxel as a Potential Mechanism for Its Antiangiogenic Effects: Potentiation by Cox-2 Inhibition

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Paclitaxel has antiangiogenic properties, but the mechanisms for the enhanced sensitivity of endothelial cells (ECs) to this drug are not established. The aims of our study were to compare the distribution of paclitaxel into ECs with other cell types, to assess the effects of low doses of paclitaxel on Cox-2 expression and to determine the combined effects of paclitaxel and Cox-2 inhibitors on angiogenesis *in vitro* and in patients with cancer. Upon exposure to low (5 nM) concentrations of [^{3}H]-paclitaxel, uptake of radioactivity was more than 5 times higher in ECs than other cell types. Exposing human umbilical vein ECs to low nanomolar (1–50 nM) concentrations of paclitaxel enhanced Cox-2 expression more than 2-fold, as measured by ELISA. Combined treatment with paclitaxel and the Cox-2 inhibitor NS-398 resulted in increased antiendothelial effects as compared to each agent alone. To assess the biologic effects of the combined treatment *in vivo*, 4 cancer patients were treated with a prolonged intravenous infusion of paclitaxel (10 mg/m^2/day) and the Cox-2 inhibitor celecoxib (400 mg p.o. BID), and plasma angiogenic activity and drug levels were measured. The treatment was well tolerated, providing steady-state concentrations of paclitaxel in plasma near 10 nM and potent plasma anticndothelial effects were observed. These findings suggest that antiangiogenic effects of paclitaxel may be due its preferential accumulation in ECs. Low dose paclitaxel in combination with a Cox-2 inhibitor is an attractive antiangiogenic and antitumor strategy that deserves further evaluation in clinical trials.

Key words: angiogenesis; metronomic chemotherapy, paclitaxel; Cox-2 inhibitors; plasma angiogenic activity; cancer

Angiogenesis is a key feature for tumor growth and progression.[1,2] Many chemotherapeutic agents have been identified that possess antiangiogenic properties[3–6] and some are being formally evaluated in clinical trials. Among these compounds are the microtubule-interfering agents vinblastine, paclitaxel and docetaxel.[3,7–14] Paclitaxel is a microtubule-polymerizing agent that is widely used in the treatment of solid tumors. In addition to its known cytotoxic effects, it inhibits endothelial cell migration, invasion, capillary tube formation and proliferation *in vitro* at concentrations ranging from picomolar to nanomolar, depending on the endothelial cell type and the assay conditions.[3,15,16] In addition, paclitaxel has been shown to significantly reduce microvessel density and decrease vascular endothelial growth factor production *in vivo*.[3,10,12] The mechanism(s) responsible for the exquisite sensitivity of ECs to paclitaxel have not been elucidated. Because ECs appear to be particularly sensitive to paclitaxel, we hypothesized that they may have the ability to accumulate paclitaxel to a greater degree than other cell types, which could contribute to their enhanced sensitivity to this agent.

A growing body of evidence indicates that Cox-2 plays a significant role in tumor angiogenesis and that Cox-2 inhibition suppresses tumor growth and neovascularization in different tumor types.[17–19] The contributions of Cox-2 in tumor angiogenesis include increased expression of VEGF by tumor cells; the production of the eicosanoid products thromboxane A2, PGE2 and PGI2 that can directly stimulate EC migration and growth factor induced angiogenesis and potentially inhibition of EC apoptosis by stimulation of Bcl-2 or Akt activation.[19,20] Paclitaxel has been shown to induce Cox-2 mRNA expression, increase Cox-2 protein levels and enhance PGE2 production in epithelial and tumor cell lines.[21] Cox-2 overproduction induced by paclitaxel may therefore cause the undesirable effect of indirectly stimulating angiogenesis by this mechanism. This led us to the hypothesis that the antiangiogenic effects of paclitaxel might be enhanced by Cox-2 inhibition.

This report shows that the enhanced sensitivity of ECs to low concentrations of paclitaxel may be due to their increased ability to accumulate the drug over non-ECs. It is also demonstrated that low concentrations of paclitaxel increases Cox-2 expression in ECs and simultaneous treatment with a Cox-2 inhibitor enhances the antiproliferative activity of paclitaxel against stimulated ECs. These findings were extended to the clinical setting, where patients with prostate cancer or malignant melanoma were treated with low dose paclitaxel given as a prolonged continuous *i.v.* infusion together with the specific Cox-2 inhibitor celecoxib given orally. Antiangiogenic activity was detected in the plasma specimens obtained from the patients during therapy, as measured by *in vitro* bioassays. These findings suggest that treatment with low dose paclitaxel in combination with a Cox-2 inhibitor may be an attractive clinical strategy to enhance the intrinsic antiangiogenic and antitumor effects of each agent alone.

Material and methods

Reagents

The clinical formulation of paclitaxel (Taxol®, Bristol Myers Squibb, Princeton, NJ) a 6 mg/ml (7.02 mM) solution in Cremophor EL and dehydrated alcohol, was diluted in sterile phosphate-buffered saline (PBS) to the desired concentrations and used for the bioassays. [o-benzamido-^{3}H]-paclitaxel (specific activity, 37 Ci/mmol) was purchased from Moravek Biochemicals (Brea,

Abbreviations: bFGF, basic fibroblast growth factor; Cox-2, cyclooxygenase-2; EC, endothelial cell; HMVEC-D, human microvascular endothelial cells-Dermal; HMVEC-L, human microvascular endothelial cells-Lung; HUVEC, human umbilical vein endothelial cells; PSA, prostate specific antigen; VEGF, vascular endothelial growth factor.

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CA). The Cox-2 inhibitor NS-398 (Sigma-Aldrich Research, St. Louis, MO) was dissolved in sterile DMSO (Sigma-Aldrich Research, St. Louis, MO) to a concentration of 1 mM and then diluted in sterile PBS to the desired working concentrations. The clinical formulation of celecoxib (Celebrex, Pfizer, Groton, CT) was used for the clinical trials (pure celecoxib was not available at the time of the *in vitro* studies). Matrigel (Collaborative Biomedical Products, Bedford, MA) was used at 7 mg/ml for *in vitro* angiogenesis (tube formation) assays. Basic fibroblast growth factor and vascular endothelial growth factor were purchased from R&D Systems (Minneapolis, MN). The cell proliferation reagent WST-1 (Roche, Indianapolis, IN) was used for proliferation assays. Additional solvents, reagents and chemicals were obtained from commercial sources in grades appropriate for direct use unless otherwise specified.

Cell culture

Human umbilical vein endothelial (HUVE) cells, human lung microvascular endothelial cells (HMVEC-L) and human dermal microvascular endothelial cells (HMVEC-D) were obtained from Clontech Lab, Inc. (Palo Alto, Ca) and used between passages 3 and 5. They were maintained in EGM2-MV medium (Clontech, Palo Alto, Ca). Primary human fibroblasts (IMR-90) were obtained from ATCC (Manassas, VA) and maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and antibiotics. The human renal carcinoma (786-O) and prostate cancer (PC-3) cell lines were obtained from ATCC. PC-3 cells were maintained in RPMI medium supplemented with 10% FBS and antibiotics and 786-O cells were maintained in DMEM plus 10% FBS and antibiotics. All cells were grown at 37°C in a 100% humidified incubator with 5% CO_2. Cells were grown to 80–90% confluency, harvested with trypsin, and resuspended to the cell density required for each assay.

In vitro *angiogenesis (matrigel tube formation) assay*

The matrigel tube formation assay was performed as previously described.[22] Different treatments were added to the cells before plating onto the matrigel-coated plates. After 12–16 hr of incubation, tube formation was observed through an inverted photomicroscope (Nikon, Melville, NY). Microphotographs were taken and quantitative analysis was performed as previously described.[22]

Cell proliferation assay

Proliferation assays were performed as previously described.[22] Briefly, 4×10^3 cells, suspended in endothelial basal medium supplemented with 1% FBS, were seeded into each well of a 96-well microtiter plate and treated with proliferation stimulants and active agents and incubated at 37°C for 72 hr. At the end of this period, WST-1 (10 μl) was added to each well, incubated at 37°C for 3 hr and absorbance at 450 nm was determined using a microplate reader (Bio-Rad, Hercules, CA). The experiments were performed in triplicate, and the figures presented represent the average of triplicate experiments.

Distribution of paclitaxel into cultured cells

The distribution of paclitaxel into cultured cells was determined as previously described[23,24] with modifications. Briefly, cells (1×10^5) were plated on each of 12-well plates and incubated overnight. The next day, cells were treated with ^{3}H paclitaxel and incubated for 4 hr at 37°C. After this period, cells were washed on ice with PBS 3 times and then lysed with 1.5 M NaOH. The lysate was then added to 5 ml of scintillation fluid and radioactivity was measured for 5 min. Cells treated with cold (nonradiolabeled) paclitaxel, incubated at the same time and under the same conditions were used to determine the cell number for each cell type. Total cell count per well was determined. Total cell volume was determined by multiplying the number of cells by the volume of an average cell, as estimated by measuring the average diameter of 30 cells under a microscope with ×40 magnification. Single cell volume was calculated using the formula for the volume of a sphere. This information was used to calculate the radioactive equivalent concentration of paclitaxel within the cells. Experiments were performed in triplicate wells and repeated at least twice.

Determination of endothelial Cox-2

HUVE (2×10^6) resuspended in EBM-2 medium with 5% FBS and antibiotics and plated in 60 mm plates were treated with paclitaxel and incubated at 37°C for different time periods. At each time period, the cells were lysed with RIPA buffer (Boston Bioproducts, Ashland, MA) and protease inhibitor cocktail tablets (Roche Diagnostics, Indianapolis, IN). Protein concentration of the cell lysates was determined by the BCA™ protein assay (Pierce, Rockford, IL), following the manufacturer's recommendations. Cox-2 determination of the lysate (100 μL) was performed using a commercially available ELISA kit (Alpha Diagnostic International, San Antonio, TX), following the manufacturer's recommendations. Cox-2 concentration of the lysates was corrected for total protein concentration and presented as the average of triplicate measurements and standard deviations.

Patients and treatment plan

Protocols to perform pilot phase II studies of low dose, continuous *i.v.* infusion paclitaxel and oral celecoxib for patients with hormone refractory prostate cancer and melanoma were reviewed and approved by the local Institutional Review Board. Results of these studies will be formally reported elsewhere, when completed. Four patients (all the patients available at the time of this report) are presented here, and the data presented on the patients is focused on the translational studies. All patients signed a written informed consent document as a condition of participation in the study. Patients who failed standard therapy and had preserved organ function were eligible. The minimum time from any prior treatment and entry into these protocols was 1 month. The dose of paclitaxel was based on a phase I trial of low dose, continuous infusion paclitaxel in combination with radiation therapy.[25] Paclitaxel was delivered through a central catheter as a continuous *i.v.* infusion at a rate of 10 mg/m^2/day for 6 consecutive days without interruption, followed by 1 day without treatment. Administration of celecoxib 400 mg *p.o.* twice a day at 7:00–8:00 in the morning and evening was initiated after completing the first week of paclitaxel. Treatment was continued until disease progression or unacceptable toxicity occurred.

Blood collection

Blood samples for biological assays were drawn from a peripheral vein in the arm contralateral to the site of drug administration and collected directly into citrated tubes. Sample tubes were mixed by inversion and placed on wet ice until centrifuged (3,210*g* for 10 min, 4°C). The plasma was sterilized by filtration through a 0.2 μm membrane filter (Millipore Corporation, Bedford, MA) and then stored at −70°C until assayed. Lepirudin (Aventis Pharmaceuticals, Kansas City, MO) at a concentration of 10 U/ml was added to plasma before the cell based bioassays to prevent clot formation. Serum was obtained from blood collected in serum isolator tubes. After allowing the blood to stand at room temperature for 30 min to induce clot formation, the tubes were centrifuged at 3,210*g* for 10 min and the serum filter-sterilized prior to storage at −70°C.

Blood specimens (7 ml) for drug concentration determinations were drawn from a peripheral vein in the arm contralateral to the site of drug administration and collected directly in tubes containing freeze-dried sodium heparin anticoagulant. Samples were obtained before dosing, once a week during the first month of therapy, and every other week thereafter. Sample collection was restricted to days 4–6 of each weekly infusion of paclitaxel, at least 1 hr before or after replacing the dosing solution, to insure that steady-state conditions had been achieved. In practice, the samples were obtained 6 to 8 hr after the morning dose of celecoxib was taken. Sample tubes were mixed by inversion and placed on wet ice until centrifuged (1,000*g*, 10 min, 4°C) within